

RESEARCH ARTICLE

Impact of transport conditions and underlying disease on post-stocking survival of juvenile *Lates calcarifer*

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Abstract

Diseases caused by pathogens commonly occurring in the aquatic environment or those that are non-host specific are prevalent and threaten the rapid growth of tropical aquaculture. This study investigates causes of mortality in 12 batches of newly stocked juvenile *Lates calcarifer* from three different hatcheries. Cytology based on Diff-Quik™-stained tissue and blood smears provides rapid diagnosis of possible causes of mortality, while histopathology and haematology provide a better understanding of how prolonged transport and fish with existing chronic disease are more likely to experience elevated mortality post-stocking. Our findings showed that accumulation of ammonia during prolonged transport causes extensive damage to epithelial barriers in gastrointestinal tracts and depressed immunity due to marked hypoglycaemia, predisposing fish to acute Streptococcosis. *Lates calcarifer* with chronic bacterial enteritis developed severe hypoglycaemia, had low circulating total plasma protein, and suffered high mortality within 24 hours post-stocking. Hypoglycaemia and low circulating blood proteins disrupt osmoregulation and exacerbate dehydration, which is fatal in fish in sea water. Dying *L. calcarifer* tested PCR positive for scale drop disease virus (SDDV) at 28 days post-stocking showed a 10-fold elevation of white blood cell counts, severe vasculitis, and obstruction of blood supply to major organs. Destruction of important immune organs such as spleen is a hallmark of SDDV infection that explains high incidences of opportunistic *Vibrio harveyi* infections in 61% of fish with SDDV. Overall, this study reiterates the importance of stocking disease-free fish and reducing transport stress.

KEYWORDS

ammonia, aquaculture, cytology, haematology, histopathology, transport stress

1 | INTRODUCTION

Asian seabass or Barramundi, *Lates calcarifer*, is an increasingly important tropical food fish species in Southeast Asia (Domingos et al., 2021; Tendencia, 2002; Wilkinson et al., 2008). *Lates calcarifer*

is fast growing, performs well in intensive aquaculture, and is a versatile species tolerant to freshwater, brackish, and marine rearing conditions (Jerry, 2014). Singapore is heavily reliant on juvenile fish sourced from hatcheries in neighbouring countries such as Malaysia, Indonesia, and Australia (Begum, 2022). Farms regularly experience

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mortality due to suboptimal transport conditions and underlying disease in newly stocked fish. Juvenile fish are often transported over long distances at high stocking densities (Acerete et al., 2004; McDonald et al., 1993; Sampaio & Freire, 2016; Zahedi et al., 2017). Transporting live fish in restricted volumes of water over extended periods leads to significant deterioration of water quality, resulting in marked elevations in ammonia and carbon dioxide and high bacterial load (Amend et al., 1982; Das et al., 2004; Erikson et al., 2022; Ip et al., 2001; Mielke et al., 2022; Paterson et al., 2003; Shabani et al., 2016). Ammonia is a cytotoxic compound, causing damage to the protective mucosal barrier of skin, gills, and alimentary tract (Benli et al., 2008; Chin et al., 2020; Esam et al., 2022; Portz et al., 2006) and increasing disease susceptibility (Abdullah et al., 2017; Abu-Elala et al., 2016; Ackerman et al., 2006; Carballo et al., 1995; Evans et al., 2006; Li et al., 2014; Magnadottir, 2010; Tacchi et al., 2015; Thune et al., 1993). Accumulation of carbon dioxide during transport reduces the pH of water and leads to acidosis, impairing oxygen uptake (Amend et al., 1982; Bhuiyan et al., 2022; Paterson et al., 2003). Elevated circulating hormones such as epinephrine (adrenalin) and cortisol (Barton & Iwama, 1991; Sun et al., 1992) due to transport stress cause hyperglycaemia, neutrophilia, and depressed immune-related enzymes such as lysozyme and myeloperoxidase activity (Ronchetti et al., 2018; Sadhu et al., 2014). Mass mortalities are frequently observed in fish soon after transport, which have been associated with bacterial (Arif et al., 2016; Assane et al., 2022; Mielke et al., 2022), viral (Fusianto et al., 2021; Stene et al., 2014), and parasitic (Assane et al., 2022; Kent et al., 1995) infections.

Diseases caused by bacteria commonly present in the aquatic environment such as *Vibrio* and *Streptococcus* species are frequently reported in tropical aquaculture. Although these bacterial infections are mostly opportunistic, they result in rapid onset of mortalities and significant production losses in newly stocked juvenile fish (Dong et al., 2017; Jahangiri et al., 2022). In contrast, scale drop disease caused by scale drop disease virus (SDDV) was initially observed in 100–300 g *L. calcarifer* 3–4 months after stocking into sea cages (Gibson-Kueh et al., 2012). However, with the increasing prevalence of SDDV, the disease is now reported in *L. calcarifer* up to 1.1 kg in Singapore, Malaysia, Thailand, and Indonesia (de Groof et al., 2015; Gibson-Kueh, 2012; Nurliyana et al., 2020; Senapin et al., 2019). SDDV is commonly complicated by co-infections with *Vibrio harveyi*, *Flavobacterium columnare*, *Lates calcarifer* herpesvirus, or ectoparasites such as *Lernanthropus* and *Diplectanum species* (Charoenwai et al., 2021; Domingos et al., 2021; Dong et al., 2017; Kerddee et al., 2020). Monogeneans and marine leeches are non-host-specific ectoparasites that produce large numbers of sticky eggs and are difficult to eradicate, with heavy infestations often associated with high mortalities in intensive aquaculture (Jahangiri et al., 2022; Kua et al., 2010; Ogawa, 2015). Production losses in Asian aquaculture due to parasites often exceed 30%–50%, with significant economic losses either directly from mortality caused by parasite infestations or indirectly from the cost of treatment and preventative measures (Shinn et al., 2015). The highest production cost reported

is attributed to *Benedenia seriolae* at M\$214 per annum, and overall losses may exceed billions of dollars globally (Shinn et al., 2015).

The rapid growth in aquaculture production and food security is threatened by infectious diseases. Disease outbreaks are often associated with higher cumulative mortality in the tropics due to higher temperatures favouring pathogen proliferation and transmission (Brumfield et al., 2021; Gallana et al., 2013), lack of systematic monitoring to inform management decisions, and unavailability of effective control measures. Juvenile fish are more susceptible to disease due to less developed immune system (Gomes et al., 2017; Leung & Bates, 2013). This study investigates mortality events in newly stocked juvenile *L. calcarifer* using cytology, haematology, histopathology, and PCR to identify possible causes of disease and better understand production losses.

2 | MATERIALS AND METHODS

2.1 | Background

This study is based on 12 batches of juvenile *L. calcarifer* stocked into a commercial marine floating grow-out facility in Singapore at different times, over a period of 13 months. These fish originate from three different hatcheries in Singapore, Johor, or Perak. Perak is located more than 500 km away from Singapore, such that the overland journey will take more than 10 hours. In contrast, fish transport from local Singapore and Johor hatcheries took approximately 2 h to the grow-out facility over land and/or sea in boats. These fish transportation occurred at high stocking densities of approximately 300 to 400 kg/m³, with pure oxygen supplementation. Although feed was withheld for one to two days, fish were not sedated and ammonia levels often reached greater than 8 ppm during transport. Each batch of fish generally consisted of 15,000 to 20,000 fish, which were stocked into 80-tonne flow-through tanks supplied with sand-filtered sea water.

Water quality in transport tanks and grow-out tanks was analysed (Table 2). Fish were transported in water derived from culture ponds, where sea water is diluted by rainfall over time, thus explaining the variation in salinity recorded. Total ammonia nitrogen (TAN) (ppm), nitrite (ppm), nitrate (ppm), and pH levels were tested using API test kits (API Marine kit for pH > 7.4 and API Freshwater kit for pH < 7.4), salinity using a refractometer (Kern Optics, Balingen, Germany), and dissolved oxygen (DO) concentration (mg/L), DO saturation (%), and temperature (°C) taken using a portable OxyGuard DO and temperature meter (Handy Polaris 2, OxyGuard™, Denmark). Free ammonia nitrogen (FAN) was estimated using exponential curve graphs generated to correlate FAN to TAN at different temperatures and pH values, based on conversion tables in Loh and Landos (2011) and Francis-Floyd et al. (2022) (Table 2). FAN often exceeded 0.1 ppm during transport of fish in this study.

Findings on clinically healthy juvenile *L. calcarifer* sampled from each batch at stocking are summarized in Table 3, and moribund fish collected during periods of post-stocking disease outbreaks are presented in Table 4. Disease outbreaks with daily mortality exceeding

100 to 1000 fish were observed either within 24 h or within several weeks post-stocking in this study. Moribund fish may display loss of schooling behaviour, appetite loss, body ulcerations, swollen abdomen, scale loss, or darkened body. Fish were anaesthetized with 40ppm AQUI-S™ immersion baths for blood collection, and freshly killed fish were kept chilled on ice until processed for tissue collection on the same day. Individual fish total body length (cm) and body-weight (g) were recorded for calculating condition factor (K) (Kumar et al., 2016; Solanki et al., 2013).

$$K = 100 \frac{\text{Fish bodyweight}}{\text{Total length}^3}$$

Tissue and blood smears were air-dried on glass slides and stained with Diff-Quik™ (Thermo Shandon Limited, USA) for microscopic examination. All major organs were collected into 10% phosphate-buffered formalin for histoprocessing at Diagnostic Veterinary Pathology Laboratory, Murdoch University, Australia, or Institute of Molecular and Cell Biology (IMCB), Agency for Science, Technology, and Research (A*STAR), Singapore, into haematoxylin and eosin (H&E)-stained tissue sections. Selected wax block tissues sampled at stocking ($n=7$ per hatchery) were stained with Martius Scarlet Blue (MSB) for fibrin and Perls' Prussian blue (Perls') for iron. Kidney tissues were placed in 95% ethanol and stored at -80°C until processed for molecular analysis. Stained tissue/blood smears and histology slides were examined using Olympus BX53

transmission light microscope, Digital Camera DP74, and cellSens™ Standard Imaging System (Olympus Corporation).

2.2 | Haematology

Blood glucose (mmol/L), haemoglobin concentration (g/L), haematocrit (%), total plasma protein (g/L), total red blood cell count ($10^{12}/\text{L}$), mean corpuscular volume (fL), mean cell haemoglobin (pg), mean cell haemoglobin concentration (g/L), total and differential white blood cell counts ($10^9/\text{L}$), and lymphocyte ($10^9/\text{L}$), monocyte ($10^9/\text{L}$), and heterophil counts ($10^9/\text{L}$) were determined according to methods as outlined in Chen et al. (2023).

2.3 | PCR

DNA was extracted from ethanol-fixed kidney tissues using DNeasy Blood & Tissue Kit (250) (QIAGEN, Germany) according to the manufacturer's instructions. Extracted DNA was tested by conventional polymerase chain reaction (cPCR) using published primer sequences and methods for the novel *Vibrio* spp. that cause "big belly" disease (Gibson-Kueh et al., 2021; Segers & Grisez, 2005), *Vibrio harveyi* (Pang et al., 2006), *Streptococcus iniae* (Torres-Corral & Santos, 2021), scale drop disease virus (SDDV) (Sukhontip et al., 2019), or *Lates calcarifer*

TABLE 1 gBlock gene fragments were synthesized from published primer sequences for scale drop disease virus, *V. harveyi*, and *Lates calcarifer* herpesvirus, and they were used as positive controls for conventional PCR tests.

Reference	ID primer	Primer sequences (5' > 3')	Amplicon size (bp)	Pathogen	Gene target
Sukhontip et al. (2019)	qSDDV-AF qSDDV-AR	AATGACCGAAATACGACCGAGAAC GCGGGGATCAAATGTCGTTTTG	135	Scale drop disease virus	Adenosine triphosphatase (ATPase)
Pang et al. (2006)	toxRF1 toxRR1	GAAGCAGCACTACCGAT GGTGAAGACTCATCAGCA	382	<i>Vibrio harveyi</i>	toxR
Chang et al. (2018)	LCHV_MEP_Fw LCHV_MEP_Rw	ATCACATCGTCACCTACCGC ATGGTCTGTCGCTTTCGGAG	277	<i>Lates calcarifer</i> herpesvirus	Major envelope protein

TABLE 2 Water quality during transport of juvenile *Lates calcarifer* from 3 hatcheries and in grow-out tank.

Hatchery/ grow-out tank	No. of batches	Transport duration (h)	Total ammonia nitrogen ^a (ppm)	Free ammonia nitrogen ^b (ppm)	NO ₂ (ppm)	NO ₃ (ppm)	pH ^a	Salinity (ppt)	DO (mg/L)	DO saturation ^c (%)	Temperature (°C)
Singapore	2	2	8	0.2	0	5	7.4	28–29	21.3–23.3	330.6–364.2	29–30
Perak	3	>10	8	0.01–0.1	0	5	6.3–7.2	17–31	11.6–17.6	178.5–272.1	28–31
Johor	7	2	4–8	0.1–0.2	0–1	0	6.6–7.4	15–30	5.0–14.6	155.4–350.5	25–28
Grow-out tank	–	–	0	–	0	0–5	7.6–8.0	27–31	7.82–11.8	137.1	28–29

Note: Fish were fasted 1–2 days prior to transfers. Neither sedation nor ammonia-binding additives were used during transport.

^aReadings were based on API test kit with a range for total ammonia nitrogen (TAN) of 0 to 8 ppm and for pH from 6.0 to 8.8.

^bFree ammonia nitrogen (FAN) levels were derived using an exponential curve derived from tabulated values of FAN at TAN concentrations at various pH values and temperatures in Loh and Landos (2011) and Francis-Floyd (2022).

^cPure oxygen supplementation was used during transport, leading to supersaturation of transport water.

TABLE 3 Haematology of juvenile *Lates calcarifer* from three hatcheries at stocking.

Parameters	Singapore (n=23)	Perak (n=34)	Johor (n=88)	Reference intervals (n=156)
Weight (g)	18.2±6.0	17.9±4.8	42.4±22.2	41.8±26.6
Condition factor (K)	1.2±0.1a	1.3±0.2b	1.3±0.1b	1.3±0.2
Glucose (mmol/L)	5.0±2.2a	3.8±1.9b	5.4±0.8a	2.4–11.3
Haemoglobin (g/L)	67.0±9.6b	73.8±11.6c	87.6±14.6a,*	56.0–85.0
Haematocrit (%)	25.0±4.7b	27.2±5.0b	33.6±5.7a	18.9–39.2
Total plasma protein (g/L)	68.2±8.1a	62.5±6.4b	66.7±5.0a	56.0–77.0
Total red blood cells (10 ¹² /L)	7.0±1.8	7.5±1.6b	9.3±2.0a	4.1–11.2
MCV (mean corpuscular volume) (fL)	38.2±4.6	38.4±9.2	39.8±8.4	25.8–78.1
MCH (mean cell haemoglobin) (pg)	10.5±1.8	10.4±2.3	10.1±1.7	6.5–17.4
MCHC (mean cell haemoglobin concentration) (g/L)	270.9±29.5	273.2±19.0b	263.8±43.9a	197.4–317.6
Total white blood cells (10 ⁹ /L)	49.6±30.7b	15.9±14.0c	9.5±22.7a	5.3–69.9
Lymphocyte (%)	43.4±18.7a,*	64.4±16.8b	68.2±17.2b	57.0–92.0
Monocyte (%)	24.0±12.8a	18.1±10.5b	16.2±7.8b	3.0–30.0
Heterophil (%)	32.7±16.1a,*	17.5±9.8b	15.6±13.3b	1.0–20.3
Lymphocyte count (10 ⁹ /L)	18.7±11.6b	9.5±6.0c	4.3±5.1a,*	4.7–51.4
Monocyte count (10 ⁹ /L)	12.1±9.1b	3.1±4.0c	2.3±8.7a	0.3–16.2
Heterophil count (10 ⁹ /L)	18.8±17.4b,*	3.4±5.8c	3.0±10.5a	0.6–8.4

Note: Alphabets a, b, and c represent significant differences between groups in each row ($p < .05$). Asterisk (*) represents mean blood values outside previously established blood reference intervals for clinically healthy juvenile *L. calcarifer* (Chew and Gibson-Kueh 2023).

herpesvirus (LCHV) (Chang et al., 2018) based on initial indications of disease from smear cytology or histopathology. Nuclease-free water without DNA templates served as negative controls for all PCR tests. Double-stranded synthetic DNA gene fragments (gBlocks®) manufactured by Integrated DNA Technologies were used to validate PCR or qPCR methods and in-house standards as positive controls for SDDV, LCHV, and *V. harveyi* (Table 1). In-house PCR-positive control tissues for *S. iniae* and novel *Vibrio* species of “big belly” disease used in this study were from cases previously diagnosed based on histopathology (Chew et al., 2023; Gibson-Kueh et al., 2021).

Each cPCR was performed using Taq PCR Core Kit™ (QIAGEN), in 12.5 µL final volume reactions containing 10× Taq buffer, Taq DNA polymerase (5 units/µL), dNTPs (10 mM), forward and reverse primers (10 µM), nuclease-free water, and extracted DNA (at least 20 ng/µL) or gBlocks® gene fragments (10⁵ copies/µL) for positive control, on a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) according to published cycling conditions for specific pathogens (Chang et al., 2018; Gibson-Kueh et al., 2021; Segers & Grisez, 2005; Sukhontip et al., 2019; Torres-Corral & Santos, 2021). PCR products were visualized on a 1.5% agarose using gel with a 100-bp DNA ladder (Biolabs, New England) using the Axygen® Gel Documentation System.

2.4 | Statistical analysis

Data analysis was carried out using SPSS software (IBM SPSS ver. 27). Evaluation of normality was conducted using the Shapiro–Wilk test,

and assessment of equal variances was performed using Levene's test. The selection of parametric or non-parametric test was dependent on any violations of assumption in normality and equal variances. Differences in blood parameters between the juvenile *L. calcarifer* from the three hatcheries (Johor, Perak, and Singapore) were compared using ANOVA (parametric) or Kruskal–Wallis test (non-parametric), followed by a post-hoc series of Tukey's HSD tests (parametric) or Mann–Whitney U tests (non-parametric). Total red blood cell counts of fish from the three hatchery groups showed normality with equal variances and were compared using ANOVA followed by post-hoc Tukey's HSD test. In addition, blood parameters of healthy versus diseased fish with “big belly” disease, SDDV, *S. iniae*, or marine leech were compared using between-subject Student's *t*-test (parametric) or Mann–Whitney U test (non-parametric), at $p < .05$.

3 | RESULTS

3.1 | Health status of juvenile *Lates calcarifer* from 3 different hatcheries at stocking, based on haematology and histopathology

Juvenile *L. calcarifer* from all three hatcheries were subjected to high ammonia during transport (Table 2). The journey from Perak which is located the furthest away from the grow-out facility in Singapore took more than 10 h. Blood glucose and total plasma protein (TPP) were significantly lower in fish from Perak (glucose 3.8 ± 1.9 mmol/L,

TABLE 4 Summary of findings in juvenile *Lates calcarifer* from three hatcheries during disease outbreaks post-stocking.

Source	Time post-stocking	No./size examined	Haematology	Cytology	Histopathology	PCR test	Diagnosis
Singapore	24h	19 (14.7 ± 5.1g)	↓ GLU, Hb, Hct, TPP, lymphocyte %, heterophil %, lymphocyte count, heterophil count ↑ Monocyte %, monocyte count	Cocci bacilli bacteria observed in gut smear (Figure 4a)	Severe and extensive chronic enteritis in the gut with intralosomal, clusters of large coccobacilli (Figure 4b)	n = 12/19 tested • 12 "Big belly" bacteria (+ve) • 1 <i>Streptococcus iniae</i> (+ve) • 7 <i>Vibrio harveyi</i> (+ve)	"Big belly" disease
Perak	24h	12 (17.2 ± 3.8g)	↓ Hb, Hct, TPP, total RBC count, MCHC	Abundant coccoid-shaped bacteria in blood smear, indicative of bacterial septicaemia (Figure 4c)	High numbers of cocci bacteria in spleen, kidney, and liver (Figure 4d).	n = 6/12 tested • 6 <i>S. iniae</i> (+ve)	<i>Streptococcus</i>
Johor	>1 month	28 (81.2 ± 99.8g)	↓ GLU, Hb, Hct, total RBC count, MCHC, lymphocyte % ↑ Total WBC count, monocyte %, heterophil %, lymphocyte count, monocyte count, heterophil count	High numbers of monocyte and heterophils in blood smear	Spleen with depleted RBCs, extensive infarction, and multifocal haemorrhage described by Gibson-Kueh et al. (2012)	n = 28 • 28 Scale drop disease virus (+ve) • 17 <i>V. harveyi</i> (+ve) • 1 "Big belly" bacteria (+ve) • 1 <i>Lates calcarifer</i> Herpesvirus (+ve)	Scale drop viral disease
		10 (65.2 ± 17.5g)	↓ Hb, Hct, TPP, total RBC count, MCHC, lymphocyte %, heterophil % ↑ Total WBC count, monocyte %, lymphocyte count, monocyte count	Severely depleted of red blood cells and often showed abundant monocytes or monocytes in blood smear	Spleen is severely depleted of RBCs (Figure 5d)	No PCR conducted	Marine leech infestation

Note: ↓ represents a significant depression, while ↑ represents a significant elevation of blood parameters in *L. calcarifer* from each hatchery source during disease outbreaks compared to values at stocking ($p < .05$).

Abbreviations: GLU, glucose; Hb, haemoglobin; Hct, haematocrit; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell; TPP, total plasma protein (TPP); WBC, white blood cell.

TPP 62.5 ± 6.4 g/L) than in Singapore (glucose 5.0 ± 2.2 mmol/L, TPP 68.2 ± 8.1) and Johor (glucose 5.4 ± 0.8 mmol/L, TPP 66.7 ± 5.0 g/L) ($p < .05$) (Table 3, Figure 1). Histopathology of juvenile *L. calcarifer* from all three hatcheries showed acute degenerative changes with spongiosis, oedema, and necrosis in gills, stomach, intestine, kidney, and liver, in association with prolonged exposure to significantly high ammonia during transport. Fibrin deposits were extensive in the stomach, intestine, pyloric caeca, kidney, and liver tissue sections (MSB stain), particularly in fish from Perak (Figure 2a–c). Fibrin plaques associated with acute spongiotic or necrotic epithelium were observed in the gastrointestinal tracts (including anterior gut, pyloric caeca, and stomach) and kidney (Figure 2). To a lesser degree, scattered fibrin plaques were observed within the liver, spleen, and brain. Splenic iron deposits in haemosiderophages and melanomacrophage centres (Perl's stain) were observed in Perak fish with the longest exposure to ammonia, suggesting haemolysis and increased toxicity from prolonged exposure to ammonia (Figure 2d).

Fish from Singapore hatchery had underlying chronic granulomatous enteritis, or “big belly” disease, based on histopathology and cPCR. Additionally, they exhibited depleted liver lipid stores (Figure 3c,d) and significantly lower haematocrit ($25.0 \pm 4.7\%$) (Table 3). Condition factor of fish from Singapore was significantly lower ($1.2 \pm 0.1K$) than fish from Perak ($1.3 \pm 0.2K$) and Johor ($1.3 \pm 0.1K$) ($p < .05$). Total white blood cell ($49.6 \pm 30.7 \times 10^9/L$), heterophil ($18.8 \pm 17.4 \times 10^9/L$), lymphocyte ($18.7 \pm 11.6 \times 10^9/L$), and monocyte ($12.1 \pm 9.1 \times 10^9/L$) counts were significantly higher in fish from Singapore than in Perak and Johor at the time of stocking ($p < .05$) (Table 3, Figure 1).

3.2 | Findings in juvenile *Lates calcarifer* from 3 different hatcheries sampled during disease outbreaks post-stocking

Table 4 summarizes the findings in diseased *L. calcarifer* sampled during periods of increased mortality post-stocking, based on cytology, histopathology, and PCR. Both batches of *L. calcarifer* from Singapore hatchery had underlying chronic granulomatous enteritis or “big belly” disease detected at the time of stocking and suffered high mortality within 24 h post-stocking. All three batches of Perak *L. calcarifer* that experienced a >10 -h transport journey suffered high mortality due to *Streptococcus iniae* within 24 h post-stocking (Figure 4). Four of seven batches of *L. calcarifer* from Johor experienced elevated mortality 28 days post-stocking and tested PCR positive for scale drop disease virus (SDDV). Histopathology of SDDV-infected *L. calcarifer* observed in this study is as previously described, with multifocal to extensive splenic infarcts and glomerulonephritis (Domingos et al., 2021; Gibson-Kueh et al., 2012). Two out of the same four batches that tested PCR positive for SDDV suffered heavy infestations with marine leech (Figure 5). Fifty-eight per cent of Singapore *L. calcarifer* with “big belly” disease ($n = 7/12$) and 61 per cent Johor *L. calcarifer* with SDDV ($n = 17/28$) had concurrent infections with and were tested PCR positive for *V. harveyi* (Table 4).

The difference in haematological parameters of clinically healthy *L. calcarifer* at stocking and diseased *L. calcarifer* is summarized in Tables 5 and 6. Total WBC and monocyte counts were significantly elevated in moribund *L. calcarifer* that tested PCR positive for chronic diseases such as “big belly” disease and SDDV (Table 5).

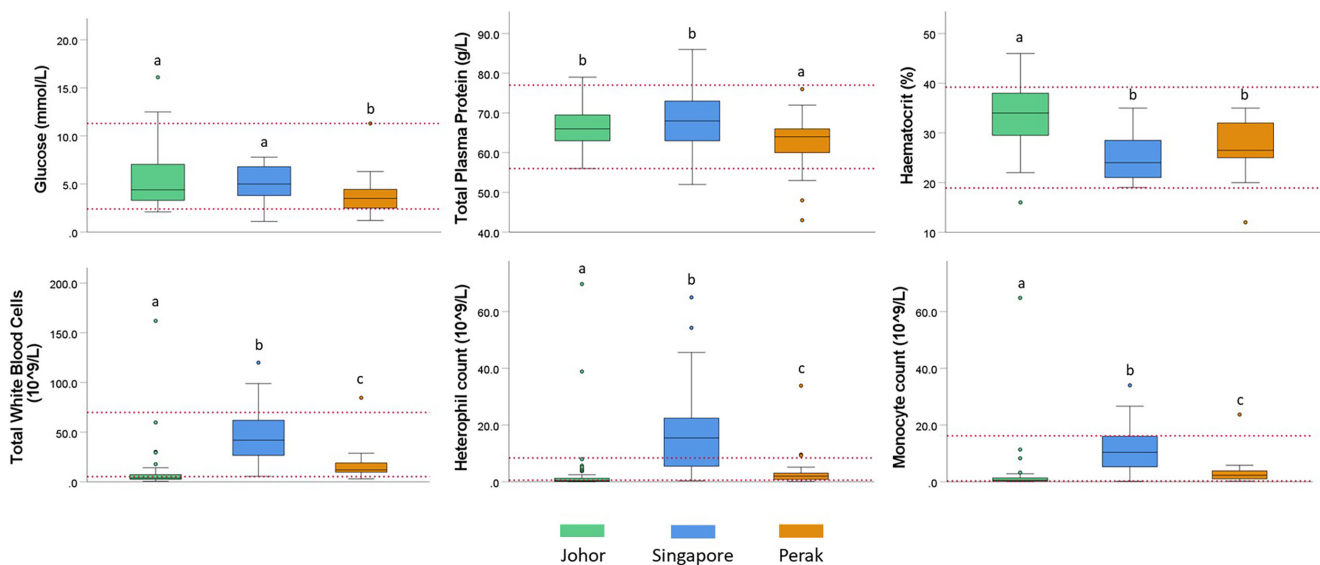


FIGURE 1 Total WBC, heterophil, and monocyte counts were significantly elevated in juvenile *Lates calcarifer* from Singapore hatchery with chronic granulomatous enteritis or “big belly” disease at stocking. Blood glucose and total plasma protein were significantly lower in fish from Perak after a 10h transport journey. Haematocrit was significantly lower in fish from Singapore with “big belly” disease and in Perak fish exposed to high ammonia with indications of haemolysis based on the increased presence of haemosiderin in macrophages and melanomacrophage centres in spleen. Alphabets a, b, and c represent significant differences between groups ($p < .05$). Red dotted line indicates blood reference intervals established for clinically healthy *L. calcarifer* by Chew and Gibson-Kueh (2023).

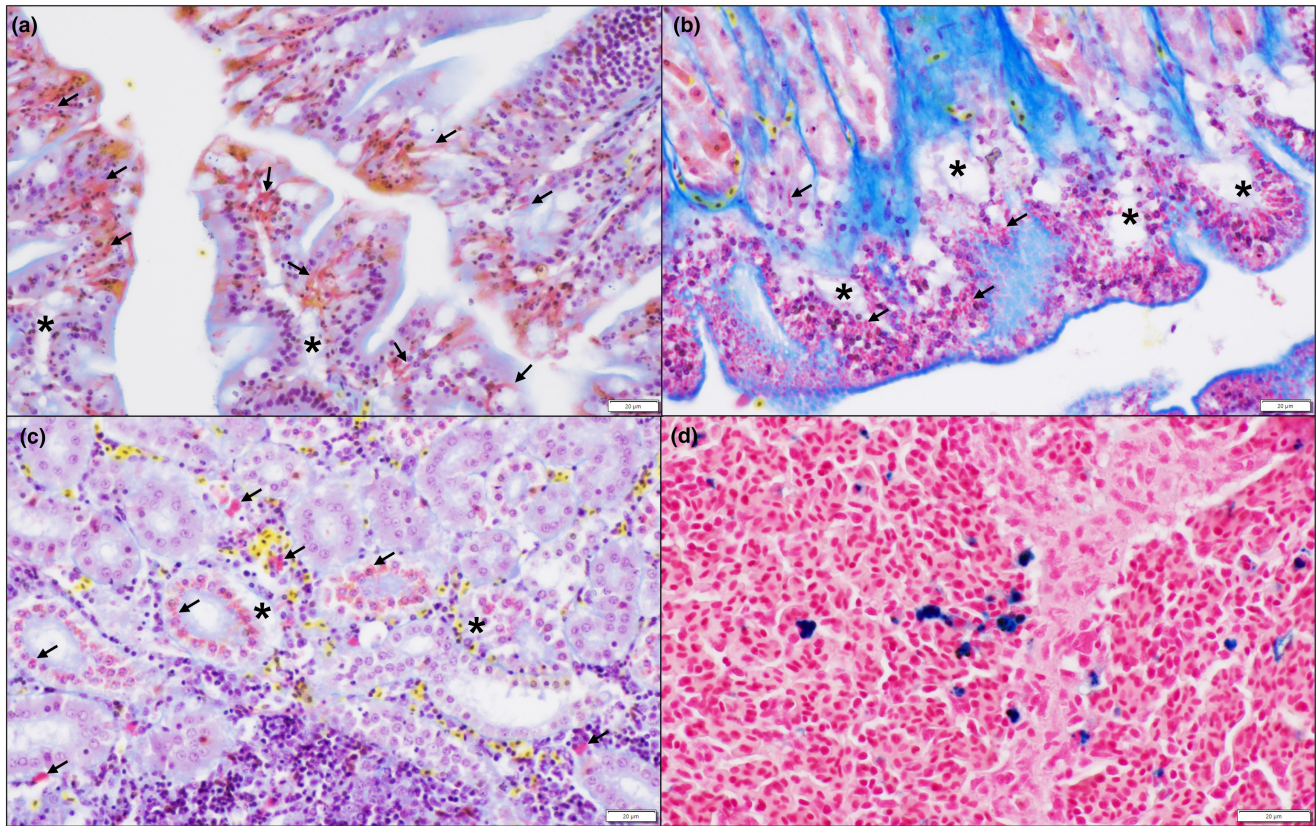


FIGURE 2 Martius Scarlet Blue stain. The presence of plaques or islands of red staining fibrin (arrows) is observed in association with acute degenerative changes and necrosis (pyknosis, karyorrhexis) in mucosal epithelium of (a) intestines, (b) stomach, and (c) renal tubules of kidney. Note the separation of epithelium from basement membranes (*). Perl's stain (d) spleen. Iron deposits stained blue in haemosiderophages and pigmented macrophage centres suggest haemolysis, presumably due to prolonged exposure to high ammonia levels. Fibrin plaques within blood vessels or adhered to degenerated or necrotic tissues indicate antemortem tissue damage (Laurens et al., 2006). Polymerization of fibrinogen may not occur in all vertebrates including fish and look more homogenous as in this study (Petersen et al., 2018). Scale bars: a–d, 20 μ m.

SDDV PCR-positive fish showed a marked 10-fold elevation of WBC total counts, lymphocytosis, monocytosis, and heterophilia (Table 6). Acute infection with *S. iniae* was associated with significant depression of haemoglobin, haematocrit, total plasma protein, total red blood cell counts, all white blood cell type counts or panleucocytosis, and MCHC (Tables 5 and 6, Figures 6 and 7).

4 | DISCUSSION

This study investigates cause of mortality in 12 batches of newly stocked juvenile *L. calcarifer* from three different hatchery sources and provides insights into improving survival post-stocking. Cytology based on Diff-Quik™-stained tissue and blood smears was invaluable in providing rapid diagnosis of possible causes of mortality and informing upstream molecular analysis (Figure 4), while histopathology and haematology provided a better understanding of why prolonged transport, exposure to ammonia, and fish with existing chronic diseases such as “big belly” disease were more likely to experience elevated mortality events post-stocking. Ammonia toxicity is primarily due to its un-ionized form (NH_3) or

FAN, which is influenced by salinity, temperature, pH, oxygen saturation, and duration of exposure (Parvathy et al., 2023; Randall & Tsui, 2002). Perak fish were exposed to FAN at or exceeding 0.1 ppm for extended periods greater than 10 hours during transport (Table 2). Prolonged exposure to 0.13 ppm FAN or higher has been reported to cause mortality in both freshwater fish (Hasan & Macintosh, 1986; Thurston et al., 1981; Zhang et al., 2012) and marine fish (Rodrigues et al., 2014; Sampaio et al., 2002; Thurston et al., 1978).

In this study, Perak fish suffered heavy mortality due to *Streptococcus iniae* septicemia within 24 h after stocking. Extensive spongiosis and necrosis of the gastrointestinal tracts were observed in fish from Perak and previously reported in association with ammonia toxicity (Cao et al., 2021; Ding et al., 2020; Liu et al., 2023; Luo et al., 2023; Qi et al., 2017; Wang et al., 2021). Fish in sea water constantly drink to combat dehydration, and this increases the exposure of gut to toxic ammonia during transport (Dehler et al., 2017; Thrall et al., 2012; Whittamore, 2012). Disruption of epithelial barriers of the gut (Perera et al., 1997), nares (McNulty et al., 2003), gills (McNulty et al., 2003; Varga et al., 2022), and skin (Bromage & Owens, 2002; Xu et al., 2007) increases risks to invasion

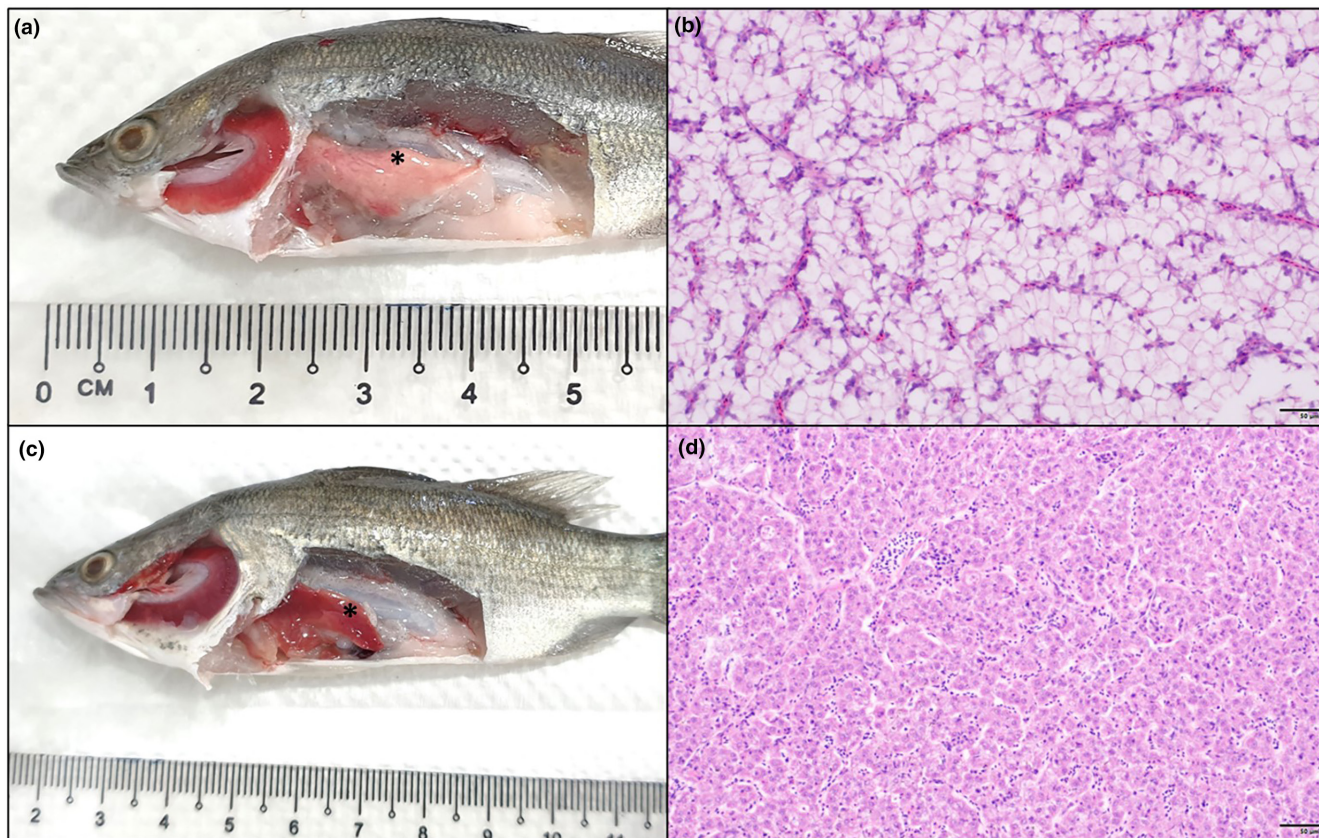


FIGURE 3 Juvenile *Lates calcarifer* sampled at stocking. (a) Pale creamy liver (*) and (b) hepatocytes with wispy staining eosinophilic cytoplasm (H&E) indicative of good lipid storage in fish from Perak. (c) Lipid-depleted dark red liver (*) in Singapore fish with “big belly” disease and (d) amphophilic staining hepatic parenchyma (H&E). Scale bars: b and d, 50 µm.

by pathogens commonly present in water (Benli et al., 2008; Esam et al., 2022; Jerônimo et al., 2022; Parvathy et al., 2023). Floating marine fish farms in Singapore are located in close proximity to land, and this predisposes farmed fish to infection by bacteria that are commonly associated with surface run-offs, such as *Streptococcus* sp. (Awate et al., 2023; Chou & Lee, 1997; Creeper & Buller, 2006; Dinh-Hung et al., 2023; Leigh et al., 2018). Bacterial loads may increase several folds during transportation of live fish and increase risks of bacterial infections (Bhuiyan et al., 2022; Gamoori et al., 2023; Hossain et al., 2021). Mean total plasma protein (TPP) levels were significantly lower in fish from Perak (62.5 ± 6.4 g/L) than in Singapore and Johor at stocking ($p < .05$; Table 3). In addition to gut damage, *L. calcarifer* exposed to FAN during transport in this study exhibited multifocal renal tubular necrosis like that described in other studies (Abd Elnabi et al., 2018; Mangang & Pandey, 2021; Zhang et al., 2018). Protein-losing enteropathy and renopathy due to acute damage to gut epithelia and kidney tubules may explain the lower TPP in Perak fish (Junior et al., 2018; Thrall et al., 2012).

In addition to prolonged ammonia exposure, all transported *L. calcarifer* in this study were subjected to high dissolved oxygen saturation (155.4%–364.2%) due to unregulated pure oxygen injection in tanks during transport. Injecting pure oxygen during live fish transport is a common practice, as transported fish are often stocked at high densities (Crosby et al., 2006). Similarly, high stocking

densities, like those observed in this study (300–400 kg/m³), have been reported during fish transport for grow-out in various species. For instance, African catfish (*Clarias gariepinus*) have been stocked at densities as high as 500 kg/m³ (Manuel et al., 2014), while rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*O. kisutch*) have been transported at densities ranging from 300–420 and 240–360 kg/m³, respectively (Harmon, 2009; Piper et al., 1982). Water supersaturated with oxygen could potentially cause hyperoxia, particularly in cases of prolonged exposure (Harmon, 2009). Supersaturation of gas in water could cause “gas bubble disease” (GBD) in fish, where gas bubbles form in the bloodstream, tissue, and organs such as gills and can cause mortality (Cao et al., 2020; Ou et al., 2016). Oxygen saturation levels of 136% have been reported to cause mortality in salmonids (Machova et al., 2017). *Lates calcarifer* in this study did not show any clinical signs of GBD, possibly due to the relatively short duration of exposure or because *L. calcarifer* is inherently more tolerant (Machova et al., 2017; Skov et al., 2013; Weitkamp et al., 2003). GBD was observed in juvenile Atlantic salmon (*Salmo salar*) 14 days after exposure to oxygen saturation of 160% (Espmark et al., 2010). In contrast, juvenile turbot (*Scophthalmus maximus* L.) could readily adapt to moderately DO-supersaturated water conditions (147%–223%) with no negative impact on their health (Person-Le Ruyet et al., 2002). Juvenile Atlantic cod (*Gadus morhua*) are tolerant of moderate levels of nitrogen gas supersaturated water of up to 105%

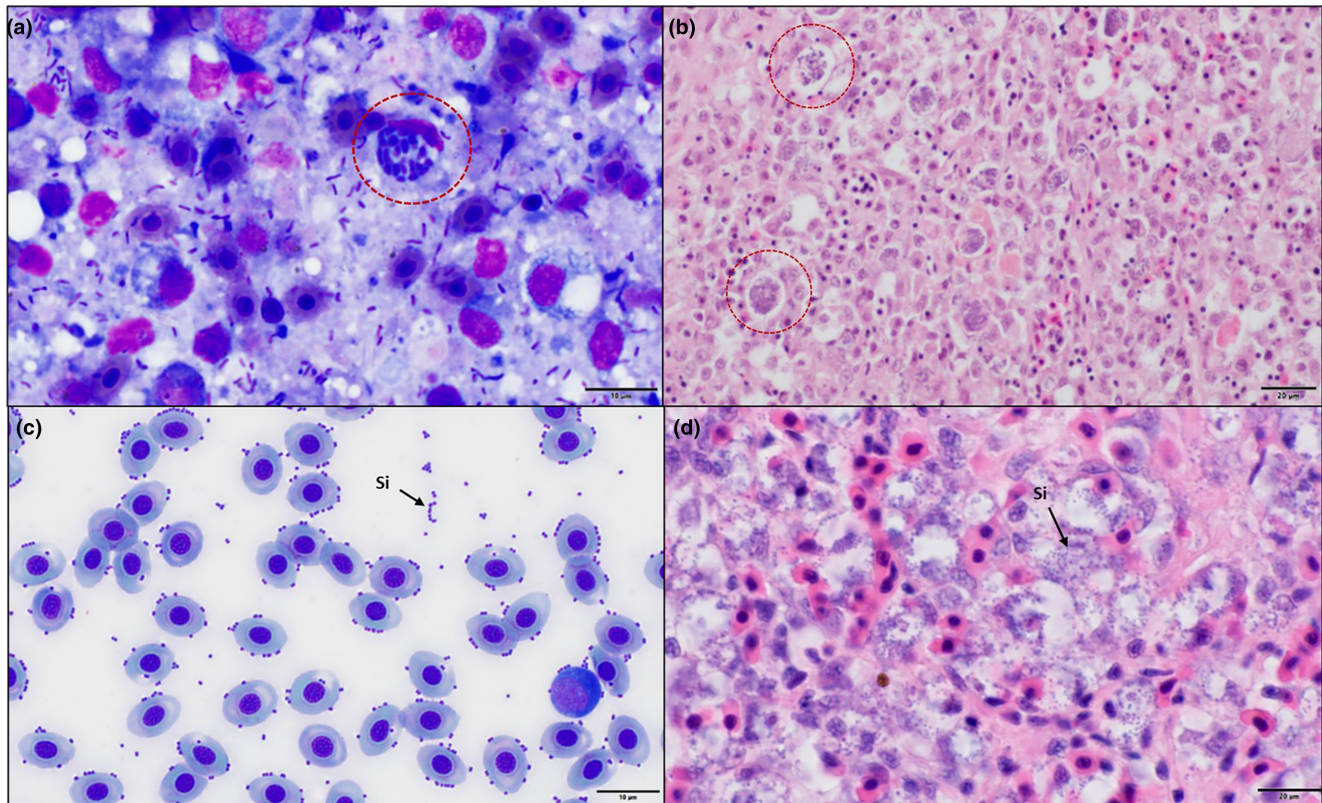


FIGURE 4 Diff-Quik™-stained blood and tissue smears offer a quick diagnosis, over their corresponding H&E-stained tissue sections from diseased *Lates calcarifer*. Clusters of coccobacillary bacteria (encircled) in *L. calcarifer* with “big belly” disease in (a) gut smear and (b) intestines with severe chronic granulomatous enteritis. Abundant coccoid-shaped bacteria, *Streptococcus iniae* (Si) in (c) blood smears, are often observed in pairs or chains and (d) spleen. Scale bars: a and c, 10 μm; b and d, 20 μm.

(Gunnarsli et al., 2008). Oxygen supersaturation could cause oxidative stress (Sampaio & Freire, 2016), affect immune functions, and predispose to disease (Chen et al., 2023).

Lates calcarifer with *S. iniae* infection 24h post-stocking exhibited a significant decrease in mean blood haemoglobin, haematocrit, red blood cell counts, and TPP levels, and blood glucose below detectable levels (Table 5). These haematological alterations are similarly reported in other fish species infected with *S. iniae* (Barham et al., 1980; Foo et al., 1985; Kim et al., 2021; Martins et al., 2011). Splenic iron deposits observed in melanomacrophage centres and haemosiderophages (Perl's stain) in Perak fish (Figure 2d) suggested haemolysis, which has been previously observed in ammonia toxicity (Xu et al., 2021; Zou et al., 2023). Carbon has been reported to appear in splenic macrophages of fish 24h after injection, such that haemosiderin can be expected to be taken up by resident macrophages in spleen in a similar time frame (Agius & Roberts, 2003; Ferguson, 1989; Lamers & Parmentier, 1985). The apparent lack of an inflammatory response or leucocytosis in response to acute *S. iniae* septicaemia may be related to severe hypoglycaemia (Figure 7). Extreme hypoglycaemia is expected to reduce the ability of cells including immune cells to generate ATP to fuel their metabolic function (Calder et al., 2007; Maclver et al., 2008).

Singapore *L. calcarifer* with underlying “big belly” disease or chronic granulomatous enteritis that suffered high mortality

within 24h post-stocking had severely depleted liver lipid stores, significantly lower mean condition factor as compared to fish from other hatchery sources, low blood glucose and TPP levels. Dehydration due to disruption of osmoregulation from hypoglycaemia and extensive gut damage from chronic enteritis (Grosell, 2006; Whittamore, 2012) can be fatal in fish in sea water (Gibson-Kueh et al., 2021; Rash & Lillywhite, 2019). Circulating blood proteins contribute to maintaining the osmotic pressure of circulating blood, and decreased proteins exacerbate dehydration in fish in sea water (Andreeva, 2019; Hankins, 2006). Transport of Singapore *L. calcarifer* with an underlying chronic “big belly” disease precipitated opportunistic bacterial infections, with kidney of seven of 12 fish tested PCR positive for *V. harveyi* and one fish for *S. iniae*. These fish which subsequently succumbed to “big belly” disease and co-infections with *V. harveyi* or *S. iniae* showed elevated mean total white blood cell ($40.5 \pm 27.5 \times 10^9/L$) and monocyte ($30.7 \pm 23.9 \times 10^9/L$) counts (Table 6), as reported in other bacterial diseases in fish (Martins et al., 2008, 2009; Ranzani-Paiva et al., 2004; Sebastião et al., 2011).

Four of seven batches of *L. calcarifer* from Johor with elevated mortality rates 28 days post-stocking and tested PCR positive for SDDV showed extreme leucocytosis, characterized by a 10-fold elevation of total WBC counts, lymphocytosis, monocytosis, and heterophilia (Table 6). Obliteration of blood vessels due to severe

TABLE 5 Haematology of *Lates calcarifer* suffering disease outbreaks post-stocking compared to clinically healthy fish at stocking.

Source	Disease	Sample size (n)	K	GLU (mmol/L)	Hb (g/L)	Hct (%)	TPP (g/L)	RBC ($10^{12}/L$)	MCV (fL)	MCH (pg)	MCHC (g/L)
Singapore	Healthy	23	1.2±0.1	5.0±2.2a	67.0±9.6a	25.0±4.7a	68.2±8.1a	7.0±1.8	38.2±4.6	10.5±1.8	270.9±29.5
	"Big belly" disease	19	1.2±0.2	1.2±0.6b,*	50.3±23.0b	17.8±7.8b,*	59.2±14.7b	6.0±1.2	49.7±0.6	12.8±0.4	283.3±53.3
Perak	Healthy	34	1.3±0.2	3.8±1.9	73.8±11.6a	27.2±5.0a	62.5±6.4a	7.5±1.6a	38.4±9.2a	10.4±2.3	273.2±19.0a
	<i>Streptococcus iniae</i>	12	1.2±0.1	Below detectable level	43.7±9.4b,*	20.9±4.8b	46.6±5.5b,*	4.7±1.3b	49.3±18.1b	10.4±3.8	209.6±11.4b
Johor	Healthy	88	1.3±0.1a,x	5.4±0.8x	87.6±14.6a,x	33.6±5.7a,x	66.7±5.0a	9.3±2.0a,x	39.8±8.4	10.1±1.7	263.8±43.9a,x
	Marine leech	10	1.0±0.2b	3.6±2.2	41.3±24.3b,*	18.6±9.4b,*	44.2±17.0b,*	4.2±2.3b	45.1±15.2	8.6±4.5	196.8±72.7b,*
	Scale drop viral disease	28	1.2±0.2y	2.8±2.5y	54.0±14.7y,*	23.0±6.5y	63.3±12.5	6.0±0.8y	48.8±16.2	11.3±3.4	234.6±53.2y
Reference interval	—	—	2.4–11.3	56.0–85.0	18.9–39.2	56.0–77.0	4.1–11.2	25.8–78.1	6.5–17.4	197.4–317.6	—

Note: Alphabets "a and b" or "x and y" indicate significant differences between healthy and diseased groups in each column ($p < .05$). Asterisk (*) represents mean blood values outside previously established blood reference intervals for clinically healthy juvenile *L. calcarifer* (Chew and Gibson-Kueh 2023).

TABLE 6 White blood cell counts in juvenile *Lates calcarifer* sampled during disease outbreaks post-stocking compared to clinically healthy fish at stocking.

Source	Disease	Sample size (n)	Total WBC count ($10^9/L$)	Lymphocyte (%)	Monocyte (%)	Heterophil (%)	Lymphocyte ($10^9/L$)	Monocyte ($10^9/L$)	Heterophil ($10^9/L$)
Singapore	Healthy	23	49.6±30.7	43.4±18.7a	24.0±12.8a	32.7±16.1a,*	18.7±11.6a	12.1±9.1	18.8±17.4a,*
	"Big belly" disease	19	40.5±27.5	13.7±11.4b	75.8±12.6b,*	10.4±9.9b	8.0±4.4b	30.7±23.9*	1.8±1.3b
Perak	Healthy	34	15.9±14	64.4±16.8a	18.1±10.5	17.5±9.8a	9.5±6.0a	3.1±4.0a	3.4±5.8a
	<i>Streptococcus iniae</i>	12	7.7±4.9	77.0±14.9b	12.7±10.0	10.3±7.4b	5.4±2.8b	1.3±1.5b	1.0±1.2b
Johor	Healthy	88	9.5±22.7a,x	68.2±17.2a,x	16.2±7.8a,x	15.6±13.3a	4.3±5.1a,x	2.3±8.7a,x	3.0±10.5x
	Marine leech	10	73.4±47.9b,*	39.2±22.6b,*	55.2±26.6b,*	5.6±7.1b	21.6±18.9b	48.2±41.1b,*	3.5±5.4
	Scale drop viral disease	28	112.7±93.6y,*	33.6±16.7y,*	53.6±19.6y,*	12.9±12.6	33.8±15.1y	54.6±65.4y,*	24.4±35.2y,*
Reference interval	—	—	5.3–69.9	57.0–92.0	3.0–30.0	1.0–20.3	4.7–51.4	0.3–16.2	0.6–8.4

Note: Alphabets "a and b" or "x and y" indicate significant differences between healthy and diseased groups in each column ($p < .05$). Asterisk (*) represents mean blood values outside previously established blood reference intervals for clinically healthy juvenile *L. calcarifer* (Chew and Gibson-Kueh 2023).

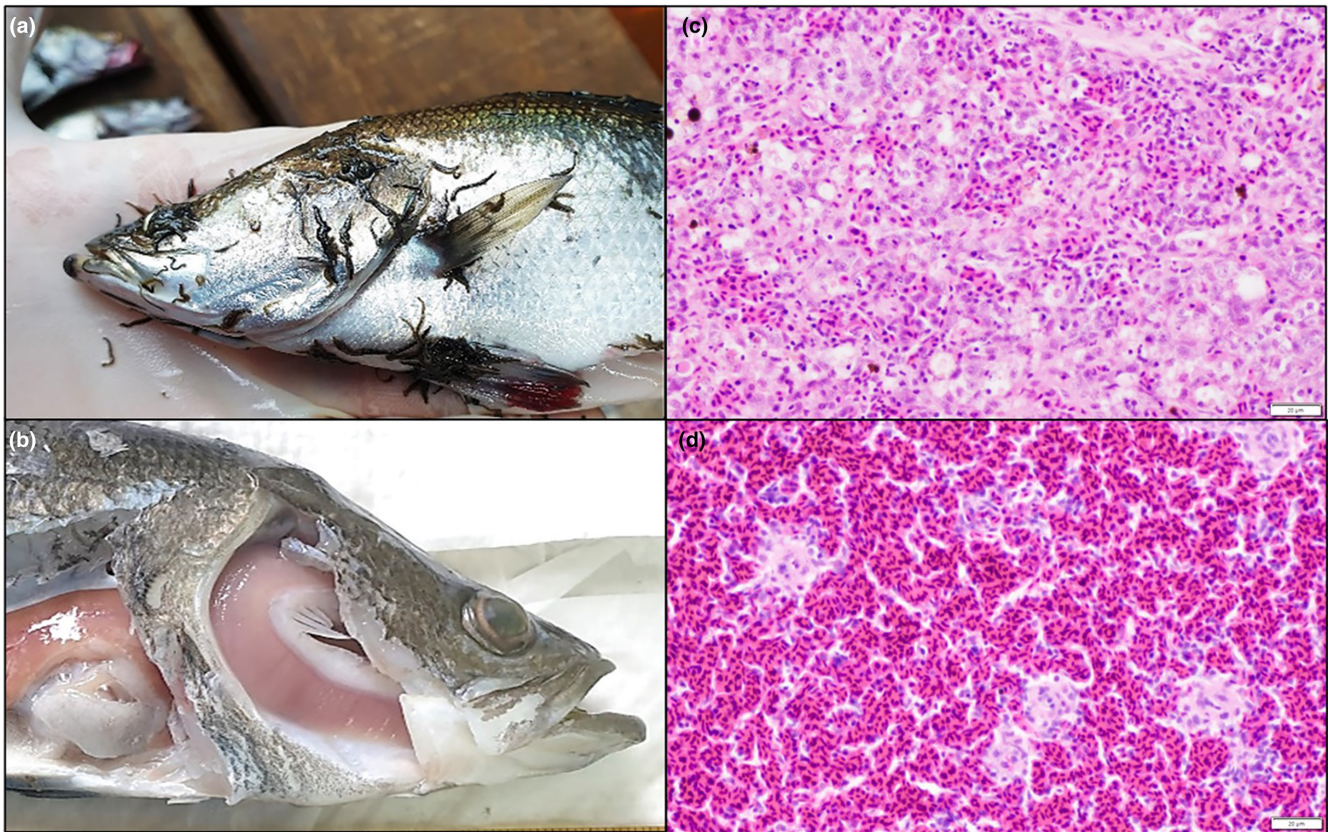


FIGURE 5 (a) Juvenile *Lates calcarifer* heavily parasitized by blood-sucking marine leeches, with (b) pale gills and severe anaemia with low haematocrit (9%). H&E spleen tissue sections (c) in leech-infested fish, with marked depletion of RBC population, and (d) in clinically healthy fish with good RBC population. Scale bars: c and d, 20 µm.

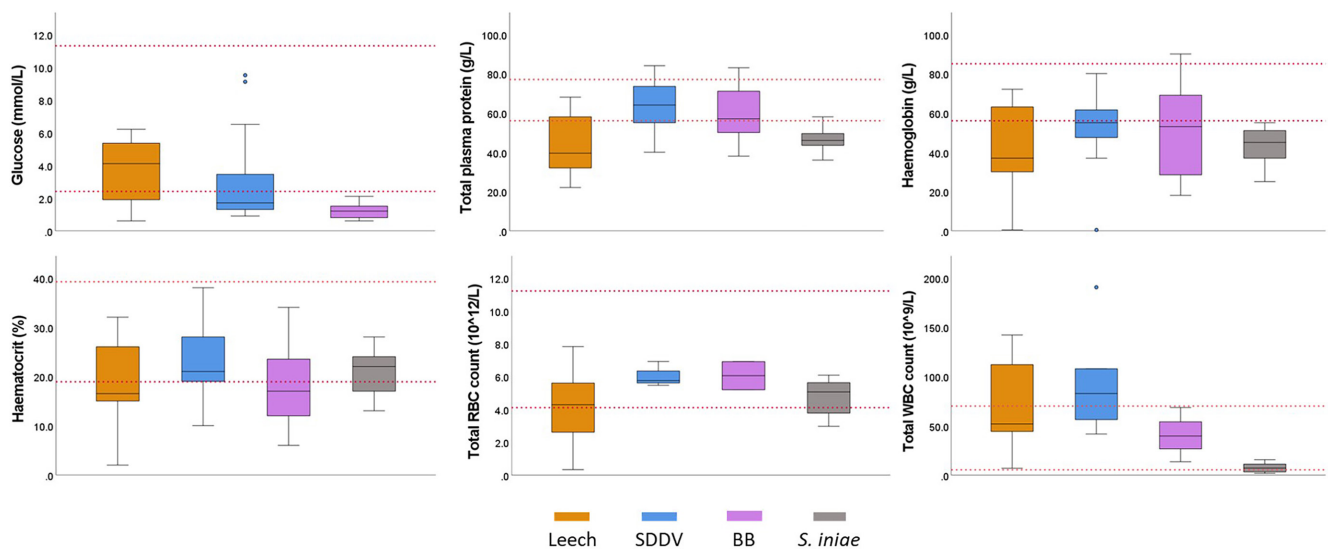


FIGURE 6 *Lates calcarifer* with marine leech infestations, scale drop disease virus (SDDV), “big belly” disease (BB), and *Streptococcus iniae* infection (*S. iniae*) showed depression of blood glucose and haemoglobin concentration, with blood glucose reaching undetectable levels in fish with *S. iniae* infections. Total WBC counts were elevated in fish with leech infestations and SDDV but depressed in *S. iniae* infections. Depression of TPP and total RBC counts was most marked with blood-sucking marine leech infestations. Red dotted line indicates blood reference intervals established for clinically healthy *L. calcarifer* by Chew and Gibson-Kueh (2023).

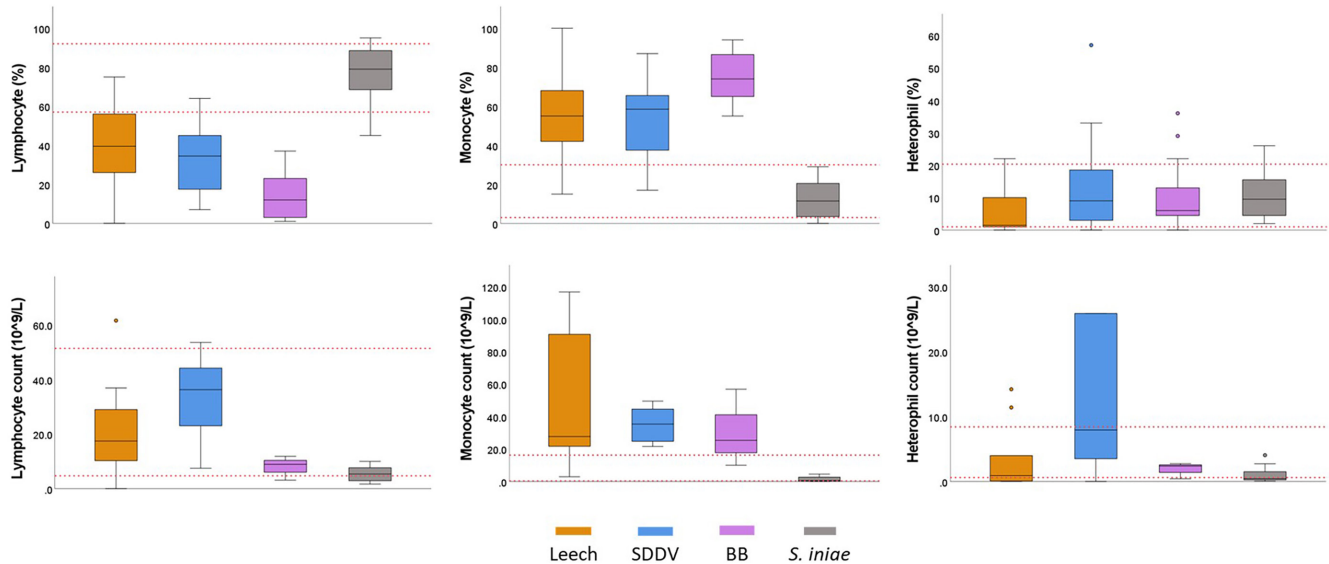


FIGURE 7 Monocyte counts are elevated in *Lates calcarifer* with marine leech infestations, scale drop disease virus (SDDV), and “big belly” disease (BB), while markedly elevated heterophil is observed only for SDDV. The apparent lack of an inflammatory response to *Streptococcus iniae* (*S. iniae*) may be related to severe hypoglycaemia. Red dotted lines indicate blood reference intervals previously established for healthy *L. calcarifer* (Chew and Gibson-Kueh 2023).

chronic inflammation that results in infarcts and destruction of important immune organs such as spleen and osmoregulatory barriers of skin are hallmarks of SDDV infections (Domingos et al., 2021; Gibson-Kueh et al., 2012). Immune depression from splenic infarcts could explain why 61 per cent of diseased Johor *L. calcarifer* with SDDV ($n=17/28$) were tested PCR positive for *V.harveyi* in this study. *Vibrio harveyi* is an opportunistic marine pathogen often reported in fish with SDDV (Domingos et al., 2021; Dong et al., 2017; Lai et al., 2023; Qiao et al., 2017).

Johor *L. calcarifer* with leech infestation exhibited the lowest mean condition factor ($1.0 \pm 0.2K$) and significantly lower levels of blood haemoglobin, haematocrit, TPP, total red blood cell count, mean corpuscular haemoglobin concentration, and lymphocyte percentages (Figure 6, Table 5). These haematological alterations are attributed to the heavy infestation of leeches and their blood-feeding activity (Celik & Aydin, 2006; Munro et al., 1992; Ogawa et al., 2007). Fish hosts with a weakened condition are more prone to ectoparasites (Poulin, 2013; Rohde et al., 1995; Shvydka et al., 2018). *Zeylanicobdella arugamensis* is the most commonly reported parasitic leech species affecting farmed fish in Brunei (Azmeiy et al., 2020), Indonesia (Afifah et al., 2021; Mahasri et al., 2020; Suyanti et al., 2021), Malaysia (Chu et al., 2013; Kua et al., 2010; Ravi & Shariman Yahaya, 2017), and Philippines (Cruz-Laciera et al., 2000). Similar to this study, *Z. arugamensis* infestations have been reported to cause secondary bacterial infections from bite wounds and elevated white blood cell counts (Kua, 2006; Kua et al., 2010; Ogawa et al., 2007; Suyanti et al., 2021). Poor tank hygiene encourages the proliferation of marine leeches in closed fibreglass tank systems, leading to heavy infestations weeks or months after stocking (Kua et al., 2010). Regular tank surface cleaning and prophylactic formalin treatment have been

recommended to manage leech infestations in closed systems (Cruz-Laciera et al., 2000).

5 | CONCLUSION

This study reiterates the importance of stocking disease-free fish and reducing transport stress to increase post-stocking survival. Our findings show that prolonged exposure to toxic free ammonia nitrogen (FAN) during transport can cause extensive damage to epithelial barriers in gastrointestinal tracts and depressed immunity due to marked hypoglycaemia, predisposing fish to invasion by pathogens commonly present in the aquatic environment. Simple haematological tests for blood glucose and total plasma protein are useful in assessing the impact of underlying disease on affected fish, as for established reference blood values in cultured *L. calcarifer* by Chew and Gibson-Kueh (2023). Hypoglycaemia secondary to chronic disease or prolonged transport compromises immune function against invading pathogens and increases susceptibility to disease. Successful disease management cannot be made solely on pathogen detection. Haematology is useful in complementing histopathology and smear cytology as decision-making tools for better disease management in aquaculture.

AUTHOR CONTRIBUTIONS

This study was designed and conceived by Xian Zhe Chew and Susan Gibson-Kueh. Xian Zhe Chew conducted the field sampling, blood analysis, and molecular laboratory work. Histopathological data analysis was carried out jointly by Xian Zhe Chew and Susan Gibson-Kueh. Molecular PCR test, positive control validation, and analysis were conducted by Xian Zhe Chew, Maura Carrai, and Xueyan Shen.

Xian Zhe Chew and Susan Gibson-Kueh wrote, reviewed, and edited the manuscript. All authors provided final approval for publication while agreeing to be responsible for the work's integrity and content.

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CONFLICT OF INTEREST STATEMENT

All authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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