Assessment of the *in vitro* survival of the Infectious Salmon Anaemia Virus (ISAV) under different water types and temperature

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Abstract

Infectious salmon anaemia (ISA) is an important viral disease affecting farmed Atlantic salmon caused by ISA virus (ISAV). A deeper understanding of the biophysical properties of ISAV is needed to design more efficient control measures. The aim of the study was to assess the *in vitro* survival of ISAV under varying water types and temperature. Multiple aliquots of four ISAV strains were dispensed in four water types and held at 5, 10, 15, and 20ºC. An aliquot was sampled every 7 to 15 days to assess viability of the virus and the viral titre was measured. The differences between survival rates of the ISAV genotypes were not significant but the differences with varying temperature and water types were statistically significant. A decrease in the survival rate of ISAV was observed when the temperature increased. ISAV could survive for extended periods in freshwater at low temperature but the survival of ISAV was greatly decreased in seawater notwithstanding water temperature. This study has shown that temperature and water type play an important role in the *in vitro* ISAV survival.

Introduction

Infectious salmon anaemia virus (ISAV) of the family Orthomyxoviridae is an important pathogen that causes disease in Atlantic salmon (*Salmo salar*) aquaculture worldwide (Kawaoka et al., 2005). Optimal *in vitro* growth temperature for ISAV ranges from 10 to 15°C, decreasing at 20°C, while it is completely inhibited at 25°C or higher (Falk et al., 1997). Within a pH ranging from 5.7 to 9.0 the virus remains stable, while it is rapidly inactivated by pH levels below 4.0 after 30 min (Danneviget al., 1995). However, contradictory published data exists with certain studies reporting that the virus remains infectious for 14 days at 4°C and for 10 days at 15°C in cell culture supernatant (Falk et al., 1997) while others have shown that ISAV survives for 4 months at 4°C in seawater (Rimstad and Mjaaland, 2002), or that it keeps its infectivity in seawater for 20 h at 6°C (Nylund et al., 1994). Factors that affect the survival of orthomyxoviruses in water are complex (Hinshaw et al., 1979). Dilution effects and the presence of

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chemical and biological antiviral agents affect virus survival unfavorably (Shuval et al., 1971), though aggregation and adsorption of virus to particulate materials can be beneficial to survival. Therefore, it can be speculated that orthomyxoviruses have a relatively good survival rate in water, thereby assisting with horizontal transmission. A deeper understanding of the biophysical properties of ISAV is needed to design more efficient measures for ISA disease control. The aim of the study was to assess the *in vitro* survival of ISAV under different ranges of water salinity, temperature and virus genotypes.

Material and methods

Virus strains

Four ISAV isolates identified as A1 (genotype HPR5, clinical case PM-3205-07); A2 (HPR35, PM-1302-08); A3 (HPR7b, PM-2752-08) and A4 (HPR15b, AY-301-08) were obtained from ISA affected Atlantic salmon during 2007-2008. The genotype of each isolate was determined by sequencing the highly polymorphic region (HPR) of segment 6 of the ISAV genome. Isolation and replication of the virus was perfomed in the Atlantic Salmon Kidney cell line (ASK, ATCC CRL2747), cultured in 75 cm^2 flasks at 20ºC in L15 media (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, USA). Viral growth was identified by a direct visual assessment of the cytopathic effect (CPE) and confirmed by TaqMan® real time RTPCR (Snow et al., 2006). The isolates were stored in liquid nitrogen (-196°C) until processing.

Virus replication and titration

Immediately prior to the *in vitro* experiments, all virus isolates were inoculated onto ASK monolayers (96 well plates) at 90% confluence and incubated at 15ºC for 14 days. The viral supernatants were filtered using a 0.45 μm cellulose acetate membrane (Nalgene®, Thermo Fisher Scientific, USA) and viral titres calculated by end point dilution using the Spearman-Karber method (Hubert, 1984).The initial titers were $10^{7.25}$ $TCID_{50}$ mL^{-1} (ISAV-A1), $10^{7.5}$ $TCID_{50}$ mL^{-1} (A2), $10^{7.25}$ $TCID_{50}$ mL^{1} (A3), and 10^{7} $TCID_{50}$ mL^{1} (A4).

Survival assays

Natural seawater (SS) and freshwater (FF) were used to simulate marine and lake/river phase waters, respectively. Additionally, a seawater and freshwater 2:1 v/v mixture (SF) and a freshwater and seawater 2:1 v/v solution (FS) were used to simulate brackish conditions. Both SS and FF samples were collected near production sites free from ISA and tested by RTPCR and cell culture prior to use in the experimental study. Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, USA) with HEPES (5,9 g/L), Phenol Red (0,01 g/L), antibiotic-antimycotic mix (Penicillin 100 UI/ml; Streptomycin 100 μg/ml; Amphotericin B 0,25 μg/ml) supplemented with 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific, USA) was used as control solution. The different water types were treated with HEPES 5.9 g/L and Phenol Red 0.01 g/L to give a pH value around 7.1. The saline concentration (SS: 30.84 g/kg; SF: 21.01; FS: 10.84; FF: 0.06), conductivity (SS: 47.46 mhos; SF: 34.43; FS: 18.27; FF: 0.11) and dissolved solids (SS: 23.72 g/L; SF: 17.71; FS: 9.1; FF: 0.05) were measured using a multiparameter tester (model HI9828, Hanna Instruments®, Romania). Water was filtered using an acetate cellulose membrane (0.45 μm) free of surfactants (SFCA Nalgene®) and was sterilized at 121ºC for 20 min. Multiple aliquots of each virus were dispensed in SS, SF, FS, FF and

control solution and held at 5, 10, 15 and 20ºC as follows; A series of tubes containing 9 mL of different water types and control solution, were inoculated with 1 mL of each viral strain with a titre value standardized to $1x10^7$ $TCID_{50}$. An aliquot was sampled every 7 to 15 days to assess viability of the virus as measured by viral end point titration, resulting in 22 readings per genotype/water type/temperature (1,408 observations) and its respective controls (352 observations) over 7 months in duplicate (3,520 measurements). Mean viral titers values of the duplicates were used (1,760).

Statistical analysis

Viral survival was defined as the interval from viral inoculation until it reached an efficient reduction of the viral load (a 99.99% reduction compared to the initial load), a criterion used by American Society for Testing and Materials (ASTM, 2002). The Kaplan-Meier survival curve and the median survival time were estimated, and then a log-rank test was applied for evaluation of the differences between survival times of different test conditions. To assess the association between temperature, salinity and the genotype survival time, an accelerated failure time (AFT) model was used (Wei, 1992). Five distributions for the hazard function were evaluated: Exponential (Exp), Weibull (Wei), log-logistic (LogL), lognormal (LogN) and generalized gamma (Gam), considering models with and without covariates.

Overall goodness-of-fit of the various distributions was assessed using four criteria. First, the maximized log likelihood functions of the models without covariates were compared, where the greatest value suggests a better fit (Collett, 1994). Second, the goodness-of-fit of nested models was evaluated by the likelihood-ratio statistic (Collett, 1994). Third, Akaike's Information Criterion (AIC) (Akaike, 1974), was used to compare the fit of all models. Finally, fit models with covariates were compared visually, based on the graphs of the Cox-Snell residuals plotted against the estimated cumulative hazard rate. In this plot, the distribution producing the straightest line fits the data best. The model building strategy was based on a mix of backward and forward procedures using a likelihood ratio test for assessing the goodness-of-the-fit of the nested models using a $p< 0.05$ as a statistical decision criteria. Two-way interactions and potential confounders were considered during the model building process. Statistical analysis was performed using software R V.2.14.1 (R Development Core Team).

Results

Descriptive findings

The raw data of titre values used to obtain survival curves are tabulated for each genotype and control (Appendix 1). The overall median time of ISAV survival without considering any covariates was 29 days (95% CI 29; 36) (Figure 1a). The Kaplan-Meier survival curves with 95% CI for each ISAV genotype (Figure 1b) shows that the median time of survival for ISAV genotype 1 (A1) was 32.5 days (95% CI 8; 51); for genotype 2 (A2) 32.5 days (95% CI 8; 51; for genotype 3 (A3) 32.5 days (95% CI 8; 51) and for genotype 4 (A4) 29 days (95% CI 8; 44) but the differences between genotypes were not statistically significant ($p>0.05$). For temperature comparisons, the Kaplan-Meier survival curves with 95% CI calculated that the median time of ISAV survival at 5°C was 43.5 days (95% CI 8; 72); at 10°C was 55.5 days (95% CI 8; 67); at 15°C was 32.5 days (95% CI

8; 44) and at 20°C was 25.5 days (95% CI 8; 29) (Figure 1c) and the differences were statistically significant ($p<0.05$). When comparing the data from the different water types, the Kaplan-Meier survival curves with 95% CI revealed a general trend; that the virus survival was inversely related to saline concentration. The median time of survival for SS was 8 days (95% CI 8; 15); SF was 25.5 days (95% CI 15; 29); FS was 47.5 days (95% CI 36; 67) and FF was 59 days (95% CI 36; 72) (Figure 1d) and the differences were statistically significant ($p<0.05$).

Results from AFT model

The evaluation of goodness-of-fit of nested, based on the likelihood-ratio chi-square statistic ($p< 0.001$), and not nested models (the smallest AIC) with covariates using different error term distributions (results not shown) showed that the model that best fits the data is the log-logistic. When all three factors were included in the model and after testing for confounders and two-way interactions, the final model contained two variables (water type

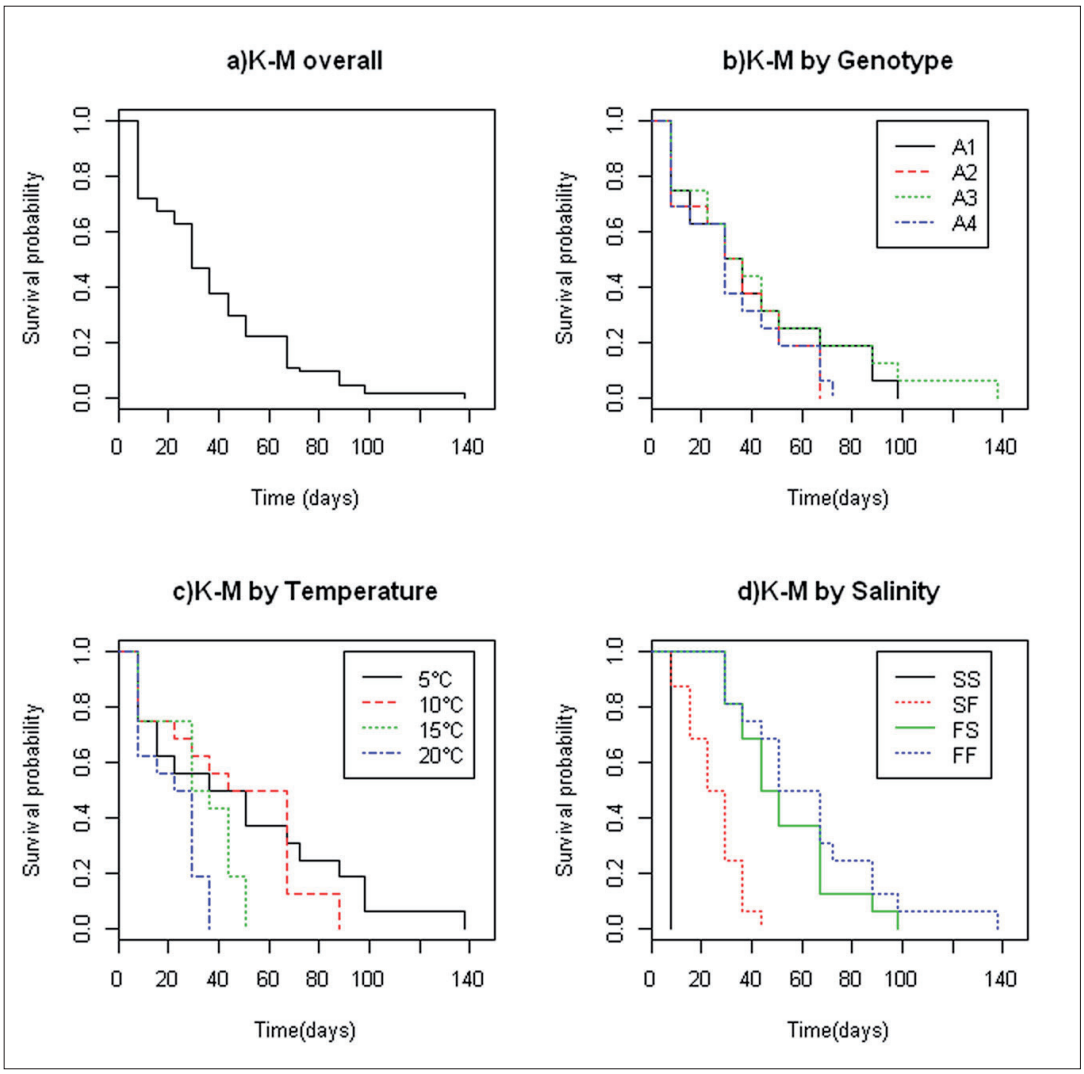

Figure 1. Kaplan-Meier survival curves (95% CI) for: a) Overall data b) Different genotypes; c) Different water temperature and d) Different water types tested in the study.

and temperature) (Table 1). Virus strains did not significantly improve the model fitness nor was there a statistically significant effect between them ($p<0.05$). Table 2 shows the time predictions of the model considering all combinations of salinity concentration and adjusted by each temperature tested in the study. Visual inspection of the Cox-Snell residuals against the estimated cumulative hazard rates shows a reasonably good fit (results not shown).

Discussion

The differences between survival rates of the ISAV genotypes were not statistically significant, but the differences between temperatures and water type were statistically significant. A decrease in the survival rate of ISAV was observed when the temperature increased. The virus could survive for extended periods in freshwater at low temperature, however, the survival of ISAV was greatly decreased in seawater independently of the water temperature. Exposing cell culture propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (Falk et al., 1997). Rimstad and Mjaaland (2002) described a three log (thousand-fold) reduction in the viral titer when ISAV was held at 4°C for 4 months. It is difficult to estimate exactly how long the virus may remain infectious in the natural environment because of a number of factors, such as the presence of particles or substances that may bind or inactivate the virus, in addition to UV irradiation and temperature as main variables (Kibenge et al., 2004).

The survival of the avian influenza viruses in the environment is influenced by temperature, pH, salinity and the presence of organic material (Stallknetch and Brown, 2009). These viruses, which are often transmitted between birds in faeces, may persist for relatively long periods in aquatic environments (Stallknetch and Brown, 2009). They appear to survive best at low temperatures and in fresh or brackish water rather than salt water (Stallknetch and Brown, 2009).

Table 1. Results from the final AFT model: hazard rate for different variables of water types and temperature with 95% confidence interval.

Variable	Category	HR	95% CI
Water	Seawater (SS)	Ref.	
Type	Seawater-freshwater - 2:1 ratio (SF)	3.00*	2.40; 3.70
	Seawater-freshwater - 1:2 ratio(FS)	6.81*	5.20; 7.60
	Freshwater (FF)	7.07*	5.80; 8.60
Temperature	5 ºC	4.11*	3.42;4.94
	10 ºC	2.51*	2.14; 2.97
	15 ºC	1.55*	1.32; 1.83
	20 ºC	Ref.	

AIC = 459.3, Log-likelyhood = -221.6, Scale = 0.163 *(P <0.001), HR = Hazard Rate

Table 2. Prediction of the ISAV survival median time (95% CI) under different water types and temperature based on the fitted model.

Water type	Temperature	Predicted time 95%CI (days)
Seawater (SS)	5 ºC	9.13 (5.03; 16.59)
	10 ºC	9.92 (5.46; 18.02)
	15 ºC	7.73 (4.26; 14.05)
	20 ºC	5.22 (2.88; 9.49)
Seawater-freshwater - 2:1 ratio (SF)	5 ºC	27.53 (15.16; 49.99)
	10 ºC	29.90 (16.46; 54.29)
	15 ºC	23.32 (12.84; 42.34)
	20 ºC	15.74 (8.67; 28.59)
Seawater-freshwater - 1:2 ratio (FS)	5 ºC	57.37 (31.59; 104.17)
	10 ºC	62.31 (34.31; 113.14)
	15 ºC	48.59 (26.76; 88.25)
	20 ºC	32.81 (18.07; 59.58)
Freshwater (FF)	5 ºC	64.59 (35.57; 117.28)
	10 ºC	70.15 (38.63; 127.38)
	15 ºC	54.71 (30.13; 99.35)
	20 ºC	36.94 (20.34; 67.08)

Avian influenza viruses are reported to persist in distilled water for more than 100 days at 28°C and 200 days at 17°C (De Benedictis et al., 2007). Various avian influenza viruses were reported to survive for four weeks at 18°C (De Benedictis et al., 2007).

In Atlantic salmon, ISA outbreaks are mainly reported in seawater cages, and only a few cases have been reported in the freshwater stage but the horizontal transmission of ISAV in freshwater has been achieved experimentally (Brown et al., 1998). Epidemiological studies suggest that ISAV can be transmitted indirectly in water and on fomites, as well as by close contact between fish (McClure et al., 2005). The virus can survive in seawater for 14 weeks (MacLeod and McBeath, 2001). This study has shown that temperature and salinity play an important role in the survival of ISAV. It also suggests that ISAV would be present in the environment for a considerably extended period of time during the early development stages in freshwater and that this may be facilitating the horizontal transmission in field conditions and determining the ISAV carrier hosts. Knowledge

of the quantitative effects of these parameters should assist with epidemiological studies on ISAV and in the development of control strategies, particularly in deciding on the correct fallowing time in seawater and freshwater stages.

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Appendix 1.The raw data of ISAV titers used to obtain survival curves. The values are means of the duplicates.

	ISAV HPR 7b															
	5º C				10º C				15º C				20º C			
Days	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**
0	1	3,5	5	5	1	3	5	5	0,5	3,5	5,5	4,5	0	3,5	4	4
8	0	2	4	4	1	2,5	3,5	4,5	0	3	5	4,5	0	2	2,5	4
15	0	3	4,5	5	0	2,5	3,5	4,5	0	2,5	3,5	4,5	0	1,5	2,5	3,5
22	0	2	3,5	4	0	2,5	4	4	0	3	3,5	3	0	0	2	2
29	0	2	3	3,5	0	2	3,5	3	0	1	2,5	3,5	0	0	0,5	1
36	0	1	3	3	0	2,5	4	4	0	1	2	3	0	0	0	0
44	0	2	3	4	0	1	4	4,5	0	1	2	2	0	0	0	0
51	0	1	2,5	3	0	1,5	3	3	0	0	0,5	1	0	0	0	0
67	0	1	3	3	0	1	2	3,5	0	0	0	0,5	0	0	0	0
72	0	1	3	3	0	0,5	2	2	0	0	0	0	0	0	0	0
77	0	1	3,5	4	0	0,5	2	3,5	0	0	0	0	0	0	0	0
88	0	1	2	3	0	0	1	2	0	0	0	0	0	0	0	0
98	0	0,5	2	2	0	0,5	0,5	1,5	0	0	0	0	0	0	0	0
110	0	0	2,5	3	0	0	0	0	0	0	0	0	0	0	0	0
117	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
127	0	0	1,5	3	0	0	0	0	0	0	0	0	0	0	0	0
138	0	0	1,5	3	0	0	0	0	0	0	0	0	0	0	0	0
156	0	0	0,5	1	0	0	0	0	0	0	0	0	0	0	0	0
163	0	0	1	2,5	0	0	0	0	0	0	0	0	0	0	0	0
169	0	0	1	2,5	0	0	0	0	0	0	0	0	0	0	0	0
179	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
225	0	0	0	1,5	0	0	0	0	0	0	0	0	0	0	0	0

ISAV HPR 35

	5º C				10º C				15º C				20º C			
Days	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**
0	2	3,5	4,5	4,5	1,5	3,5	4,5	4,5	0	3,5	4,5	5	0	2	4	4
8	0	3	3,5	3,5	1	3	4,5	4,5	0,5	3	4,5	5	0	2,5	3	3,5
15	0,5	3,5	4,5	4	0	2	4,5	4,5	0	3	3,5	3,5	0	2	2,5	3,5
22	0	2,5	3,5	3,5	0	2,5	3	4	0	1,5	3,5	4	0	0,5	2	2,5
29	0	1,5	3	3,5	0	2	3,5	3,5	0	1,5	2,5	3,5	0	0	0,5	1
36	0	2,5	3	3	0	1,5	3	3,5	0	0,5	2	3	0	0	0	0
44	0	1,5	2	3,5	0	1	2,5	4,5	0	0	0,5	2	0	0	0	0
51	0	2	3	2,5	0	0,5	3	3	0	0	0	0,5	0	0	0	0
67	0	0,5	2,5	2	0	0	1,5	1,5	0	0	0	0	0	0	0	0
72	0	0	2,5	3	0	0	1,5	2	0	0	0	0	0	0	0	0
77	0	0,5	3	4	0	0	1	2	0	0	0	0	0	0	0	0
88	0	0	1,5	3	0	0	0,5	1	0	0	0	0	0	0	0	0
98	0	0	2,5	2	0	0	0	0,5	0	0	0	0	0	0	0	0
110	0	0	0,5	1	0	0	0	0	0	0	0	0	0	0	0	0
117	0	0	1,5	1,5	0	0	0	0,5	0	0	0	0	0	0	0	0
127	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0
138	0	0	1	2,5	0	0	0	0	0	0	0	0	0	0	0	0
156	0	0	0	0,5	0	0	0	0	0	0	0	0	0	0	0	0
163	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
169	0	0	0,5	0,5	0	0	0	0	0	0	0	0	0	0	0	0
179	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
225	0	0	0	0,5	0	0	0	0	0	0	0	0	0	0	0	0

ISAV HPR 15b

	5º C				10º C				15º C				20º C			
Days	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**	**SS**	**SF**	**FS**	**FF**
0	1	3	4	4,5	1,5	5,5	5,5	4,5	0	5	5	5,5	0	4	5	4,5
8	0	3	3,5	3,5	0	3,5	4,5	4,5	0	4,5	4,5	5	0	2,5	5	5
15	0	2	3	4,5	0	2,5	4	5	0	2,5	3,5	4,5	0	1,5	3,5	3,5
22	0	2,5	4	3	0	3	4,5	4,5	0	2	3,5	4	0	0,5	2,5	2
29	0	2	3	3,5	0	3	4,5	4,5	0	1	2,5	4	0	0	1	1
36	0	1,5	3	4	0	2	3	3,5	0	1	2	3	0	0	0	0
44	0	1,5	2,5	4,5	0	2,5	3	4	0	0	1,5	2,5	0	0	0	0
51	0	1,5	2,5	3,5	0	2,5	3,5	4	0	0	1,5	1,5	0	0	0	0
67	0	1,5	2,5	2,5	0	1	2	3,5	0	0	0,5	1	0	0	0	0
72	0	0,5	3	2,5	0	0,5	2,5	4	0	0	0	0	0	0	0	0
77	0	1	3,5	3,5	0	1	2,5	3	0	0	0	0	0	0	0	0
88	0	0,5	2,5	2,5	0	0	1	1,5	0	0	0	0	0	0	0	0
98	0	0,5	2	3,5	0	0	1	2	0	0	0	0	0	0	0	0
110	0	0	2,5	2,5	0	0	0	1	0	0	0	0	0	0	0	0
117	0	0	2	3	0	0	0	1	0	0	0	0	0	0	0	0
127	0	0,5	0,5	3,5	0	0	0	0,5	0	0	0	0	0	0	0	0
138	0	0	1,5	2	0	0	0	0	0	0	0	0	0	0	0	0
156	0	0	1,5	2	0	0	0	0	0	0	0	0	0	0	0	0
163	0	0	1,5	2,5	0	0	0	0	0	0	0	0	0	0	0	0
169	0	0	1	2,5	0	0	0	0	0	0	0	0	0	0	0	0
179	0	0	1,5	2	0	0	0	0	0	0	0	0	0	0	0	0
225	0	0	0	1,5	0	0	0	0	0	0	0	0	0	0	0	0

ISAV HPR 5																
	5º C				10º C				15º C				20º C			
Days	SS	SF	FS	FF	SS	SF	FS	FF	SS	SF	FS	FF	SS	SF	FS	FF
0	2	4	4,5	4,5	1,5	3,5	4,5	4,5	0	4	5,5	4,5	0	2,5	3,5	3
8	1	3	3,5	3,5	0,5	3,5	4,5	4	0	3	4,5	4,5	0	1,5	4	3
15	0	2	4,5	3,5	0	2,5	3,5	3	0	3	3,5	3,5	0	1	3,5	3,5
22	0	1,5	3,5	3,5	0	2	2,5	4	0	1	2	3	0	0	2,5	2
29	0	2	3	4	0	2	3,5	3	0	1	3	3	0	0	1	1,5
36	0	1,5	3	3	0	1	2,5	2,5	0	0	1,5	2,5	0	0	0	1
44	0	2	2	3	0	2,5	3	3,5	0	0,5	2	1,5	0	0	0	0
51	0	1	2,5	3,5	0	1	2,5	3	0	0	0,5	2	0	0	0	0
67	0	1	2	3	0	0	1,5	2	0	0	0	1	0	0	0	0
72	0	0	2,5	2	0	0	1,5	2	0	0	0	0	0	0	0	0
77	0	0,5	2,5	3	0	0	1,5	2,5	0	0	0	0	0	0	0	0
88	0	0	1,5	1,5	0	0	0	1,5	0	0	0	0	0	0	0	0
98	0	0	2,5	2	0	0	0,5	2	0	0	0	0	0	0	0	0
110	0	0	1,5	2,5	0	0	0	0	0	0	0	0	0	0	0	0
117	0	0	2	2	0	0	0	0,5	0	0	0	0	0	0	0	0
127	0	0	1	2,5	0	0	0	0	0	0	0	0	0	0	0	0
138	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
156	0	0	0,5	2	0	0	0	0	0	0	0	0	0	0	0	0
163	0	0	0	1,5	0	0	0	0	0	0	0	0	0	0	0	0
169	0	0	0,5	1	0	0	0	0	0	0	0	0	0	0	0	0
179	0	0	0,5	1,5	0	0	0	0	0	0	0	0	0	0	0	0
225	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

CONTROL																
	PM-2752-08				PM-1302-08				AY-301-08				PM-3205-07			
Days	SS	SF	FS	FF	SS	SF	FS	FF	SS	SF	FS	FF	SS	SF	FS	FF
0	5,5	5,5	5,5	4	5,5	5	5	4,5	5,5	5,5	5,5	4,5	5	5,5	5,5	4
8	4,5	5	5	4	4	5	5	4,5	4,5	5,5	5,5	5	4,5	5	5,5	3,5
15	5	4,5	4,5	4	5	4	5	4,5	5	5	4,5	4	4,5	4	4,5	4
22	4,5	4	4	3,5	4	4,5	4,5	4	4	5	4	3,5	4	4,5	4	3,5
29	4	4,5	4	3	3,5	4	3,5	3	3,5	4,5	4	3	4	4,5	4	3
36	3,5	4	4	2	3	4	3,5	2	4	4	4	2	4	3,5	3,5	2
44	4	4,5	3,5	2	4	4,5	3	1,5	5	4,5	3	2	4	4	3	1,5
51	3,5	4	4	1,5	3,5	4	3,5	2	4	4	3,5	2	3,5	4	3,5	2
67	3	4	2,5	1,5	3	4	2,5	1	3,5	3,5	3,5	1	3	3	2,5	1
72	2,5	4	2,5	1,5	3,5	4	2,5	1	3,5	4,5	3,5	1	3,5	3	2,5	0
77	4	4	3,5	1	3,5	3,5	2	1	4	4	2,5	1,5	4	3	2,5	0,5
88	4	3,5	3	0,5	3	3,5	3	0	3	4	2,5	0	3,5	2,5	2	0
98	3,5	4	3,5	0	4	3,5	3	0	3,5	4	2,5	0	3	4	3	0
110	3	3	1,5	0	3,5	3	2	0	3,5	4	2,5	0	3	3,5	2,5	0
117	4	3	2	0	3,5	4	2,5	0	4	4	1,5	0	4	3	2,5	0
127	3,5	4	1,5	0	3,5	3,5	2,5	0	4	4	1	0	3,5	3,5	2,5	0
138	4	4	2	0	4	3,5	2	0	3,5	4	2	0	3,5	3,5	2	0
156	3	2	1	0	3	2,5	1	0	3,5	3,5	0,5	0	3	3,5	1,5	0
163	3	2	1	0	3	2,5	1	0	4	3	0	0	4	3	1	0
169	3,5	3,5	0,5	0	3,5	3,5	1	0	3	3	0	0	4	2,5	1	0
179	3	2,5	0,5	0	3	2,5	1	0	3	3	0	0	2,5	2,5	1,5	0
225	2,5	2	0	0	2,5	2	0,5	0	3	2,5	0	0	3	2,5	0,5	0