Hormone Changes in Tolerant and Susceptible Grapevine Leaves Under Powdery Mildew Infection

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Abstract
The biotrophic fungus Erysiphe necator causes powdery mildew (PM) in grapevine. Phytohormones are major modulators of defensive responses in plants but the analysis of the hormonome associated with grapevine tolerance and susceptibility against this pathogen has not been elucidated. In this study, changes in hormonal profiling were compared between a tolerant (Vitis rupestris × riparia cv. 101-14 Millardet et de Grasset) and a susceptible (Vitis vinifera cv. Aragonêz) species upon E. necator infection. Control and PM-infected leaves were collected at 0, 6, 24, 96 h post-infection (hpi), and analysed through LC-MS/MS. The results showed a distinct constitutive hormonome between tolerant and susceptible species. Constitutive high levels of salicylic acid (SA) and indole-3-acetic acid together with additional fast induction of SA within the first 6 hpi as well as constitutive low levels of jasmonates and abscisic acid may enable a faster and more efficient response towards the PM. The balance among the different phytohormones seems to be species-specific and fundamental in providing tolerance or susceptibility. These insights may be used to develop strategies for conventional breeding and/or editing of genes involved in hormonal metabolism aiming at providing a durable resistance in grapevine against E. necator.

Keywords  Erysiphe necator  ·  Grapevine  ·  Hormones  ·  Powdery mildew  ·  Tolerance  ·  Susceptibility

Abbreviations

hpi  Hours post-infection
JA-Ile  Jasmonoyl-isoleucine
JAs  Jasmonates
12-OH-JA-Ile  12-Hydroxy-JA-Ile
12-O-Glc-JA  12-O-Glucosyl-JA
12-OH-JA  12-Hydroxy-JA

Introduction
Grapevine (Vitis vinifera L.) is an important horticultural crop worldwide but susceptible to a large spectrum of pathogens (Riaz et al. 2020). Powdery mildew (PM), caused by the obligate biotrophic fungus Erysiphe necator Schw. (syn. Uncinula necator (Schw.) Burr.), is a ubiquitous disease in grapevines (Calonnec et al. 2021). The European requirements (Directive 2009/128/EC) for sustainable agriculture and the foreseeable change in pathosystems’ dynamics caused by climate changes...
make it urgent to uncover grapevine defence mechanisms against *E. necator* to develop plants with durable resistance to PM (Bois et al. 2017; Calonnec et al. 2021).

Plants display a two-layered innate immune system namely pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) with an overlapped signalling cascade to activate both site-specific and systemic defence responses (Pruitt et al. 2021). Phytohormone imbalance during infection influences the hormonal cross-communication and ultimately, the fine-tuning of the regulatory signalling network associated with PTI/ETI (Derksen et al. 2013). Salicylic acid (SA) is classically associated with resistant responses against (hemi)biotrophic pathogens, whereas jasmonic acid (JA) is implicated in resistance against necrotrophic pathogens (Glazebrook 2005; Derksen et al. 2013). Salicylic acid (SA) is classically associated with resistant responses against (hemi)biotrophic pathogens, whereas jasmonic acid (JA) is implicated in resistance against necrotrophic pathogens (Glazebrook 2005; Derksen et al. 2013). Despite being recognised as a suppressor of SA-mediated responses, reports indicate a reprogramming of the JA pathway and elicitation of resistance in rice and grapevine during infection with biotrophic pathogens (Glazebrook 2005; De Vleesschauwer et al. 2013; Pimentel et al. 2021). An emerging perspective attributes new roles in the coordination of defensive responses for other hormones such as abscisic acid (ABA). Mostly associated with abiotic stress, an ambivalent role has been described for abscisic acid (ABA) depending on the interaction and stage of defensive response (Asselbergh et al. 2008). The same holds true for auxin metabolism that has been recently associated with both resistance and susceptibility to necrotrophic and biotrophic pathogens (Kunkel et al. 2018; Coelho et al. 2019). Overall, there is a lack of knowledge of how plants coordinate their hormonal composition and signalling network to prioritise defence against pathogens (Derksen et al. 2013). In North America and East Asia, the larger centres of grapevine diversity, *Vitis* spp. exist with varying levels of resistance to PM (Riaz et al. 2020). Previous studies suggested that resistant *Vitis aestivalis* present constitutive high levels of SA in contrast to the susceptible species *Vitis vinifera* cv. Cabernet Sauvignon (Fung et al. 2008). Additionally, OMICs studies performed during *E. necator*-grapevine interaction suggested an enrichment of metabolic pathways related to hormonal biosynthesis and signalling in response to infection (Fung et al. 2008; Weng et al. 2014; Jiao et al. 2021; Pimentel et al. 2021).

In the present study, we applied LC-MS/MS technology to identify a hormone signature associated with tolerance and susceptibility of grapevine towards PM.

**Material and Methods**

**Experimental Inoculation and Sampling**

Five-year-old plants of *Vitis rupestris × riparia* cv. 101-14 Millardet et de Grasset (tolerant) and *Vitis vinifera* cv. Aragonez (susceptible) were kept in the greenhouse at Instituto Superior de Agronomia, University of Lisbon, Portugal. The genotypes were selected based on a previously large survey of hybrids and Portuguese germplasm in which cv. Aragonez exhibited strong symptoms of infection while for cv. Millardet et de Grasset no infection symptoms were noticed; this was confirmed in the present study. Aragonez is widely used in Portuguese and Spanish viticulture (named Tinta Roriz in Northern Portugal and Tempranillo in Spain) namely in Porto wine production.

Infected leaves from a field-grown *V. vinifera* cv. Aragonez served as an inoculum source. Absence of other pathogens was confirmed through observation of *E. necator* colony morphology, conidiophore and conidia under binocular and stereomicroscope (Leica, Germany). Thirty-five grapevines per species were inoculated by direct contact between the adaxial epidermis of the second–fifth leaves beneath the apex and surface of infected leaves. Mock-inoculated leaves (controls) were water-treated. Four–five biological replicates for each condition and time point were included. Based on the proposed *E. necator* infection cycle (Fung et al. 2008), control and PM-infected leaves were harvested at 0, 6, 24 and 96 h post-infection (hpi) for micro-scopical analysis or immediately frozen in liquid nitrogen and stored at –80 °C for hormonal quantification.

**Microscopical Observations**

Segments of control and PM-infected leaves were cleared in 95% ethanol and stored at 4 °C to remove the pigments. Fungal structures were stained by a trypan blue solution in lactic acid, glycerol and distilled water (1:1:1, v/v/v). Callose was stained with 150 mM dipotassium phosphate containing 0.01% aniline blue. Samples were examined twice by bright field [BX51 microscope (Olympus, Tokyo)] and fluorescence stereo microscopy [Zeiss stereo Lumar V12 (Oberkochen, Germany)].

**Hormonal Profiling**

Approximately, 30 mg of control and PM-infected leaves were freeze-dried at –40 °C for 3 days. Dry material was extracted with 1.5 mL methanol containing 60 ng D₅-SA (Santa Cruz Biotechnology, USA), 60 ng D₆-JA (HPC Standards GmbH), 60 ng D₆-abscisic acid (ABA) (Santa Cruz Biotechnology) and 12 ng D₅-jasmonoyl-isoleucine (JA-Ile) (HPC Standards GmbH) as internal standards. Then samples were processed as previously described (Pimentel et al. 2021). Phytohormone analysis was performed by LC–tandem mass spectrometry (MS/MS) as in (Heyer et al. 2018) on an Agilent 1260 series HPLC system (Agilent Technologies) coupled to a tandem mass spectrometer API5000 (SCIEX, Darmstadt, Germany), as described...
(Heyer et al. 2018) and adapted for grapevine (Pimentel et al. 2021). For quantification of the remaining compounds the individual response factors (RF) were determined by analysing a mixture of the particular compounds with either D₆-JA-Ile (for JA-Ile derivatives; RF all 1.0), D₄-SA (for SA-glucoside; RF 1.0), or D₆-JA (for OPDA; RF 0.5) at the same concentrations (Dávila-Lara et al. 2021).

**Statistical Analysis**

Statistical analysis was performed by applying a Shapiro–Wilk test to evaluate the normality, followed by Dixon’s Q-test to identify outliers. Significance on the hormone content at 0 hpi was determined with a Student’s t-test, whereas significance at 6, 24 and 96 hpi was evaluated with ANOVA-two way followed by a Tukey post-hoc test. A 95% of significance (\(p\)-value ≤ 0.05) was considered and executed on RStudio version 1.0.136 (RStudio, PBC).

**Results and Discussion**

Powdery Mildew is a disease in vineyards worldwide due to the high susceptibility of \(V. \text{vinifera}\) cultivars (Bois et al. 2017). The \(Vitis\) genus displays high genetic diversity with wild North American, Chinese non-\(V. \text{vinifera}\) and two Near Eastern \(V. \text{vinifera}\) accessions presenting different levels of resistance to PM (Jiao et al. 2021). Previous OMICs-based studies showed a reprogramming of hormonal metabolism during PM-interaction although no assessment of the respective hormonal profile was performed in leaves (Fung et al. 2008; Weng et al. 2014; Jiao et al. 2021; Pimentel et al. 2021). The present work aimed to evaluate the hormonal profile of control and PM-infected leaves of a tolerant (cv. 101-14 Millardet et de Grasset) and a susceptible (cv. Aragonez) grapevine species before and at the early stages of infection.

**Microscopic Assessment of Powdery Mildew Infection in Tolerant and Susceptible Leaves**

According to Fung et al. (2008), histological changes and fungal structures are detected in resistant and tolerant leaves at the early stage of 24 hpi. These changes were assessed in control and infected leaves at 0, 24 and 96 hpi (Fig. 1). Leaves of both species showed no evidence of fungal presence prior to infection (Fig. 1A–D). Staining of chitin and 1,3-\(\beta\)-glucan with trypan blue, at 24 hpi, allowed the validation of fungal presence, in form of spores or hyphae, at the initial stage of infection in the leaf surfaces of both species (Fig. 1E, F). Blue spots indicating cell death, an event associated with hypersensitive response to avoid progression of fungal penetration (Derkson et al. 2013), were observed in a higher amount in susceptible species (Fig. 1E, F). Presence of secondary hyphae and development of a functional haustorium were reported at 24 hpi in PM-infected leaves of resistant \(V. \text{aestivalis}\) and susceptible \(V. \text{vinifera}\) cv. Cabernet sauvignon (Fung et al. 2008). Nevertheless, we could not identify the differentiation of appressorium or secondary hyphae due to the density of mycelium in tolerant species (Fig. 1F). Reinforcement of cell wall by depositing papillae enriched with callose is triggered to slow down the invasion and enclose the haustorium in a toxic environment (Asselbergh et al. 2008). Staining of 1,3-\(\beta\)-glucan with aniline blue evidence the sites of fungal penetration in epidermal cells of both species and hyphae presence related to those sites (G, H). All images were acquired with 10×or 40×magnification in BX51 (Olympus, Tokyo) and Zeiss stereo Lumar V12 (Oberkochen, Germany). C, callose; Hy, hyphae; HR, hypersensitive reaction; My, mycelium; S, spores.

![Fig. 1] Figure 1 Microscopic assessment of Powdery Mildew (PM) on grapevine leaves. Tolerant \(Vitis \text{rupestris} \times \text{riparia}\) cv. 101-14 Millardet et de Grasset and susceptible \(Vitis \text{vinifera}\) cv. Aragonez leaves were inoculated with \(Erysiphe \text{necator}\) and histological observations were acquired at 0, 24 and 96 h post-infection (hpi). Control leaves (0 hpi) were stained with trypan blue (A, C) or with aniline blue (B, D). At 24 hpi, under a brightfield microscope, staining of fungal cell wall constituents with trypan blue allowed the validation of ungerminated spores attached on adaxial leaf surfaces in susceptible species (E), a dense mycelium in the tolerant species (F) and hypersensitive responses in both species (E, F). Under a fluorescence microscope, at 96 hpi, staining of callose deposits with aniline blue evidence the sites of fungal penetration in epidermal cells of both species and hyphae presence related to those sites (G, H). All images were acquired with 10×or 40×magnification in BX51 (Olympus, Tokyo) and Zeiss stereo Lumar V12 (Oberkochen, Germany). C, callose; Hy, hyphae; HR, hypersensitive reaction; My, mycelium; S, spores.
of fungal penetration on leaves epidermis (Fig. 1H). Since 1,3-β-glucan is also present in fungal cell walls, identification of E. necator hyphae close to callose deposits was possible (Fig. 1G, H). Altogether, these results put in evidence successful fungal infection and defensive responses in both grapevine species.

Tolerance to Erysiphe Necator is Putatively Associated with Constitutive Higher Content of SA and IAA and Additional Induction of SA During Infection

Phytohormonal quantification in control and infected leaves at 0, 6, 24 and 96 hpi revealed a constitutive higher content of SA in tolerant species compared with susceptible species (Fig. 2). Maintenance of high SA levels was also observed during infection of tolerant species with a further accumulation at 24 hpi (Fig. 2). High constitutive levels of SA and absence of significant changes during PM-infection was described in North American resistant Vitis aestivalis (Fung et al. 2008). Genetic variability in SA metabolism may dictate differential SA-mediated responses in plants during interaction with pathogens (Derksen et al. 2013). The high constitutive SA levels in tolerant species as observed in this study can be translated into a primed physiological stage capable of faster and a more efficient response to PM-infection (Delaunois et al. 2014). This occurs since, SA per se may function as an antioxidant towards E. necator attack and induce the reprogramming of major secondary metabolic pathways related to defence responses (De Vleesschauwer et al. 2013). In fact, in susceptible cv. Carignan and cv. Cabernet sauvignon and resistant Vitis pseudoreticulata, the infection was accompanied by the accumulation of compounds synthetized by several branches of the phenylpropanoid pathway, such as resveratrol, catechins, gallic acid, lignin and anthocyanins (Fung et al. 2008; Weng et al. 2014; Pimentel et al. 2021). High SA accumulation together with activation of SA signalling was reported in elicited grapevines prior to E. necator infection (Pálfi et al. 2021). Regarding susceptible species, the lower SA content in PM-infected leaves (Fig. 2) may be eventually due to E. necator affecting SA synthesis as described for other filamentous fungi (Han et al. 2019). In leaves of susceptible Vitis vinifera cv. Cabernet Sauvignon, SA levels increased only in late infection (120 hpi), indicating a biphasic defensive response against PM (Fung et al. 2008; Jiao et al. 2021). This indicates that
early accumulation of SA is required for timely and efficient activation of grapevine defensive responses towards PM (Fig. 3). In accordance, a recent meta-analysis identified the SA signalling pathway as one conservative route integrated in a regulatory network triggered in response to PM attack in Arabidopsis, barley, grape and wheat (Sethi et al. 2021). These results also suggest that even if different species use a common defensive mechanism, the spatiotemporal activation of responses may be responsible for the output of the interaction (Fig. 3).

On the other hand, glycosylation is the main process controlling endogenous levels of free SA (Huang et al. 2018). Salicylic acid is preferably glycosylated into SA-glucoside and stored in a stable but inactive form (Huang et al. 2018). In the present study, parallel to the constitutive higher content of SA-glucoside in resistant species, further induction occurred during infection at 24 hpi (Fig. 2). The similarity to SA hormonal profiling indicates a redirection of SA to its glucoside. In Arabidopsis interaction with Pseudomonas syringae pv. tomato DC3000, glycoside forms of dihydroxybenzoic acid, another SA metabolite, accumulate and integrate a positive feedback loop to induce SA synthesis (Huang et al. 2018). This suggests a role of SA-glucoside not only in modulating SA levels but also in response to pathogens. SA-glucoside has been reported to function as a slower inducer of the oxidative burst (Kawano et al. 2004).

Associated with resistance to E. necator, SA-glucoside may function in a non-toxic and controlled way to induce the oxidative burst and trigger SA-mediated responses rather than SA (Huang et al. 2018) (Fig. 3). However, we cannot rule out that SA-glucoside accumulation is mainly involved in the regulation of free SA levels.

Auxins coordinate a plethora of growth and developmental processes in plants involving a tight coordination among biosynthesis, transport, degradation and conjugation (Kazan et al. 2009; Kunkel et al. 2018). Concerning biotic stress, it has been suggested that auxin imbalance is used by biotrophic pathogens to promote their growth and virulence or is used to modulate host defences to facilitate the progression of disease (Mutka et al. 2013; Kunkel et al. 2018). When infected with the necrotrophic pathogen B. cinerea, susceptible grapes from cv. Trincadeira exhibited an increase in IAA content and activation of auxin signalling (Coelho et al. 2019). Despite the absence of changes in IAA content, RNA sequencing of grapes of cv. Carignan indicated that IAA signalling was involved in defence against PM (Pimentel et al. 2021). In the present results and as for SA, both species presented a differential composition of IAA at the constitutive level with a higher accumulation present in the tolerant species (Fig. 2). During infection, despite the significant difference between tolerant and susceptible species at 6 hpi, IAA content maintained unchanged in response to E. necator attack (Fig. 2). Though IAA content showed a
tendency to increase in all samples at 96 hpi eventually due to abiotic stress caused by mock and fungal inoculations, the results indicate that IAA may have a role as a marker of tolerance at early stages of PM-infection eventually in combination with SA (Fig. 3). This disagrees with the previously reported antagonism between these two hormones in interactions with biotrophic pathogens (Kazan et al. 2009).

Susceptibility Against Erysiphe necator is Putatively Associated with Constitutive Higher Content of Specific Jasmonates and ABA and Additional Induction of ABA During Infection

Jasmonates, a class of lipid-derived hormones, are synthesized through the biosynthetic octadecanoid pathway. Jasmonic acid, derived from OPDA, is metabolized into several derivatives, including the bioactive form JA-Ile (Farhangi-Abriz et al. 2019). JA-Ile can be hydroxylated into 12-OH-JA-Ile and further oxidized into 12-COOH-JA-Ile or deconjugated and converted into 12-hydroxy-JA (Koo et al. 2014). In turn, JA can be directly oxidized into 12-OH-JA. From 12-OH-JA, through glycosylation, 12-O-Glc-JA is produced (Haroth et al. 2019). In this work, the susceptible species presented a higher constitutive content of OPDA, JA and JA-Ile at 0 hpi (Fig. 4). Towards biotic stress, JAs are widely associated with resistance to necrotrophic pathogens and susceptibility to (hemi)biotrophic pathogens (Glazebrook 2005). In this context, higher constitutive content of JAs in the susceptible species may be involved in its susceptibility to PM (Fig. 3). A JAs-sensitized state can be present in the susceptible species where high levels of specific JAs favour E. necator infection (Glazebrook 2005). The content and composition of JAs vary according to species and can be related with response to abiotic stresses (Farhangi-Abriz et al. 2019). This may explain the hormonal differences between both species at 6 hpi caused by mock inoculations.

The intensity and duration of JA-responses reflect primarily the free pool of JA-Ile which is controlled by the fine-tuning of JA-metabolism (Farhangi-Abriz et al. 2019). Recently, 12-OH-JA-Ile was reported to be perceived, not in a weaker manner, but similar to JA-Ile (Koo et al. 2014). The tolerant species showed a significant decrease of JA-Ile and 12-OH-JA-Ile at 6 hpi in response to PM-infection which was not observed in susceptible species (Fig. 4). On the other, in susceptible grapes, these JAs showed a tendency to increase during PM-infection suggesting a role of both JAs in susceptibility associated with both organs (Pimentel et al. 2021). However, the role of the lesser known 12-OH-JA-Ile in promoting susceptibility against PM needs to be ascertained by comparing other susceptible and resistant grapevines.

Alongside conjugation, glycosylation modulates JAs signalling (Haroth et al. 2019). The compound 12-O-Glc-JA is reported in various plant organs and species (Miersch et al. 2008). In tomato, a sequential accumulation of 12-OH-JA and 12-O-Glc-JA in a JA-dependent manner was observed in response to wounding (Miersch et al. 2008). In Arabidopsis, mutants for enzymes responsible for JA conversion to 12-OH-JA increase resistance to B. cinerea (Farmer et al. 2019). Nevertheless, the exact role of 12-O-Glc-JA in defence is still not clear (Miersch et al. 2008; Haroth et al. 2019). Possibly, since glycoside forms are more soluble, 12-O-Glc-JA may be a way to transport and storage 12-OH-JA to change its bioactivity and modulate JA-mediated responses (Haroth et al. 2019). In this regard, for susceptible species, the higher content of 12-O-Glc-JA at constitutive level and at 96 hpi, with a similar trend at 6 and 24 hpi may reflect the adjustment of responses mediated by specific JAs related to susceptibility towards PM (Figs. 3, 4).

Abscisic acid has a prominent role in plant development and adaptation to abiotic stress (Asselbergh et al. 2008). As for JAs, ABA content was distinct between both species and constitutively higher in the susceptible one (Fig. 2). The content of ABA was even significant higher in control and PM-infected leaves of susceptible species at 24 hpi (Fig. 2). In parallel with the low levels of ABA in control and PM-infected leaves of tolerant species, an induction of ABA content during infection, at 24 hpi, was observed in the susceptible one (Fig. 2). The outcome of ABA as a detrimental or beneficial regulator of plant defences is still fragmented (Asselbergh et al. 2008). Pathogen manipulation of ABA biosynthetic pathway to enhance disease was reported in Arabidopsis, rice, tomato and grapevine (Asselbergh et al. 2008; Coelho et al. 2019). Either synthesized by the pathogen or host, the role of ABA as an effector molecule is related, in some interactions, to the negative regulation of SA biosynthesis and signalling (Asselbergh et al. 2008). A disease phenotype in susceptible grapevines infected with P. viticola occurred in the presence of an antagonistic correlation between SA and ABA content (Liu et al. 2016). The same hormonal profile and interaction output were suggested in the present results (Fig. 2), supporting ABA association with grapevine susceptibility towards E. necator (Fig. 3). However, in PM-infected grapes of cv. Carignan changes in ABA content were not observed (Pimentel et al. 2021) indicating organ-specific responses eventually due to ABA involvement in the onset of grape ripening (Coelho et al. 2019). Activation of ABA-mediated signalling is another
mechanism used by pathogens to suppress inducible defence responses (Asselbergh et al. 2008). Deposition of callose at penetration sites to enforce cell wall and prevent pathogen proliferation is one of the mediated processes (Asselbergh et al. 2008). The present study seems to indicate a higher callose deposition in susceptible species (Fig. 1) although the process is considered as a post-invasive defensive mechanism. Against necrotrophic pathogens, ABA and JA act in a synergetic manner to trigger the accumulation of callose (Glazebrook 2005). In the present study, higher content of specific JAs occurred in parallel with ABA accumulation (Fig. 3), but the understanding of the complex crosstalk between these hormones needs to be validated in additional susceptible and resistant species and/or mutants, and considering hormones such as ethylene, which also play a role in defence against fungal pathogens 7.

In conclusion, this study presented first insights into the hormonal composition associated with tolerance and susceptibility of grapevine against PM and enabled the assessment of hormones’ balance in determining resilience. Higher content in SA may be related to a primed state enabling a faster and more efficient response to *E. necator* in a possible

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**Fig. 4** Jasmonates content in control and Powdery Mildew-infected grapevine leaves. Tolerant *Vitis rupestris* × *riparia* cv. 101-14 Millardet et de Grasset and susceptible *Vitis vinifera* cv. Aragonez leaves were inoculated with *Erysiphe necator* and harvested at 0, 6, 24, and 96 h post-infection. Hormonal quantification (*n* = 5) in ng/g of dry weight (DW); mean ± SEM. Based on two-way ANOVA and post-hoc Tukey test: *P* < 0.05; **P** < 0.01; ***P*** < 0.001. cis-OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; OH-JA-Ile, hydroxyjasmonoyl-isoleucine; COOH-JA-Ile, dicarboxyjasmonoyl-isoleucine; JA-Glucoside, 12-O-glucoside jasmonic acid.
combination with IAA. The missing accumulation of these hormones in susceptible species in parallel with the accumulation of ABA and potentially specific jasmonates may determine the subsequent successful infection. The data also suggest that SA glycoside may play a more prominent role in PM defence besides regulating free SA levels.

Altogether, the results emphasised the importance of constitutive levels of specific hormones in determining resistance or susceptibility against PM. This knowledge once validated in other grapevine susceptible and resistant species may be used to improve the resilience of Vitis vinifera varieties to the devastating powdery mildew disease that affects dozens of other important crops.

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Author Contributions A.M.F. conceived the study. R.A., D.P., C.R. and A.M.F performed infections and sampling. Hormonal profiling was performed by A.M. Data were analysed by R.A., I.D. and H.S. R.A. drafted the manuscript. A.M.F. edited and completed it and A.M. revised it. A.M.F. agrees to serve as the author responsible for contact and ensures communication.

Declarations

Conflict of interest The authors declare the absence of conflict of interest.

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