

Comparative IVF And Embryo Development Performance of Environmentally Adapted and Conventional Cattle Genetics Under Heat Stress Conditions

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Abstract

Comparisons between environmentally adapted and conventional cattle genetics under heat stress are reported unevenly across the *in vitro* fertilization literature: some trials expose oocytes to heat only during maturation, others expose the resulting embryo only during culture, and few hold genetic group, heat-challenge stage, and endpoint definition constant within a single comparative design. This inconsistency makes it difficult to state, with laboratory-level precision, at which stage of oocyte maturation, fertilization, or embryo culture a thermotolerance advantage for environmentally adapted genetics actually operates, and whether an advantage measured at cleavage persists to blastocyst and to post-thaw survival. This paper specifies a stage-resolved comparative protocol for *in vitro* maturation, fertilization, and culture performance between environmentally adapted (*Bos indicus*-influenced) and conventional (*Bos taurus*) cattle genetics under controlled heat challenge, without reporting completed trial results. The protocol separates three challenge points, oocyte maturation, fertilization, and post-fertilization culture, and specifies cleavage rate, blastocyst rate, embryo grade, and post-vitrification survival as a linked outcome cascade rather than independent endpoints. A cellular mechanism review grounds the comparison in heat-shock protein expression, reactive oxygen species accumulation, and mitochondrial function, and a donor-management covariate set controls for body condition and nutritional status so that genetic comparisons are not confounded by donor herd management. The central proposition is that any thermotolerance advantage for environmentally adapted genetics is stage-specific rather than uniform across the *in vitro* production sequence, such that a genetic group favored at maturation is not automatically favored at culture or at cryopreservation, and that comparative trials reporting only a single pooled endpoint will misattribute stage-specific effects to genetics as a whole. Falsifiable hypotheses, quality-control standards, and a reporting checklist for stage-resolved comparative embryo trials close the paper.

Keywords

in vitro fertilization; embryo development; heat stress; Bos indicus; Bos taurus; oocyte competence; cryotolerance; comparative genetics

1 Introduction

Two cattle genetic groups respond differently, on average, to heat exposure during in vitro embryo production: *Bos indicus*-influenced genetics, associated with lower metabolic heat production and greater cellular thermotolerance, and *Bos taurus* genetics, associated with higher milk and growth performance but reduced tolerance of elevated ambient and cellular temperature (Hansen, 2009). This comparative claim is supported by a substantial literature, but that literature is heterogeneous in exactly the way that matters most for translating it into practice: studies differ in whether heat is applied during oocyte maturation, during fertilization, or during post-fertilization culture; in whether the challenge is applied to the donor animal in vivo or to the isolated gametes and embryos in vitro; and in whether the reported endpoint is cleavage, blastocyst formation, embryo grade, or post-thaw survival. A reader assembling this literature into a single claim, such as "heat-adapted genetics produce better embryos under heat stress," risks averaging across stages and endpoints that do not necessarily move together.

This paper does not report a new comparative trial. It specifies a stage-resolved protocol for comparing in vitro maturation, fertilization, and culture performance between environmentally adapted and conventional cattle genetics under heat challenge, structured so that a genetic effect at one stage can be distinguished from a genetic effect at another, and so that the outcome cascade from cleavage through post-thaw survival is tracked on the same embryos rather than reported as separate, non-comparable cohorts. Literature coverage extends through 31 December 2025. Where no authorized dataset or completed laboratory trial exists, the manuscript specifies hypotheses, protocol stages, endpoints, and validation criteria; it does not report simulated cleavage rates, blastocyst rates, or survival percentages as though they were observed.

Four questions organize the paper. First, at which cellular and molecular level does thermal injury act on the oocyte and early embryo, and does the comparative genetics literature support a stage-specific or a uniform thermotolerance advantage for environmentally adapted genetics? Second, how should a comparative in vitro production trial be designed so that maturation-stage, fertilization-stage, and culture-stage heat challenge are separable rather than confounded? Third, which outcome measures, and in what sequence, should be tracked so that a stage-specific effect is not lost by reporting only a single pooled endpoint? Fourth, what falsifiable hypotheses follow, and what donor-management and laboratory quality-control covariates must be held constant for a genetic comparison to be interpretable? The practical stakes of resolving stage specificity are not merely academic. A commercial in vitro production program deciding whether to prioritize thermotolerant donor genetics, invest in culture-stage antioxidant supplementation, or upgrade cryopreservation protocol is, in effect, being asked to place a bet on which stage of the outcome cascade carries the largest genetic-group gap, using a literature that was not designed to answer that specific question. A pooled-endpoint study reporting a higher blastocyst rate for environmentally adapted genetics cannot tell such a program whether the same donors would also show a cryotolerance advantage, or whether a conventional-genetics donor pool supplemented with a culture-stage antioxidant might close most of the observed gap at lower cost than switching donor genetics entirely. The stage-resolved design specified in Section 3 exists to make this kind of investment comparison answerable from a single, internally consistent dataset rather than from an inference stitched together across studies that differ in challenge stage,

endpoint, and laboratory protocol. The contribution is a stage-resolved comparative framework distinguishing maturation-stage, fertilization-stage, and culture-stage heat challenge; an outcome cascade linking cleavage, blastocyst formation, embryo grade, and post-vitrification survival on the same embryo cohort; a donor-management and laboratory quality-control covariate set; and falsifiable hypotheses about where in the production sequence a genetic thermotolerance advantage is expressed. As with any Q1 contribution, publication requires novelty, methodological rigor, fit to existing evidence, and transparent reporting rather than any particular venue; the protocol specified here is designed to meet those requirements while leaving empirical performance claims to a future trial conducted under appropriate laboratory and institutional animal-care oversight. A further imbalance in the existing literature compounds the stage-conflation problem. Much of the published comparative work reports whole-animal, in vivo outcomes, such as pregnancy rate after natural service or embryo transfer, rather than isolating the embryo's own developmental performance from the recipient's contribution to the eventual outcome. In vivo pregnancy rate is the commercially decisive endpoint, but it is also the endpoint furthest removed from the cellular mechanisms reviewed in Section 2, and a genetic-group difference in pregnancy rate could arise from embryo-level thermotolerance, from recipient-level uterine receptivity, or from an interaction between the two that a whole-animal design cannot decompose. Isolating the embryo as the unit of analysis, as this protocol does, sacrifices some external validity relative to a whole-animal trial in exchange for the ability to attribute an observed difference specifically to the oocyte and embryo rather than to the combined and inseparable effect of embryo and recipient.

This paper is deliberately narrower in scope than a herd-level fertility study. It does not model whole-animal pregnancy outcome, which depends on recipient uterine and endocrine factors addressed elsewhere in this research program; it isolates the laboratory-level performance of the oocyte and embryo themselves, from collection through cryopreservation, as the unit of comparison. This narrower scope is a deliberate design choice, not an oversight: whole-animal pregnancy outcome confounds embryo quality with recipient readiness, and comparative genetics claim about the embryo cannot be isolated from a recipient-level trial without controlling for recipient variation that a laboratory-level design avoids by construction.

2 Cellular and physiological bases of thermal injury

Heat stress affects mammalian reproduction through several converging cellular pathways rather than a single mechanism, and cattle reproduction is among the best-characterized systems for this convergence (Hansen, 2009). Elevated temperature during oocyte maturation disrupts meiotic spindle organization and chromosome alignment, reduces cumulus-oocyte complex expansion, and increases the proportion of oocytes arrested before completing maturation; direct heat challenge applied to bovine embryos in vitro was shown to impair cleavage and blastocyst development as early as the initial experimental work on this question three decades ago (Ryan et al., 1992), and heat challenge applied during the peri-implantation window continues to reduce embryonic survival even when maternal systemic condition is otherwise controlled (Kasimanickam et al., 2021). Seasonal heat stress in vivo compounds these direct cellular effects with altered ovarian steroidogenesis and reduced estrus expression, and hormonal treatment strategies have been proposed as partial, not complete, mitigations for the resulting fertility loss (De Rensis et al., 2015). The physiological cost of heat is distributed across the full reproductive sequence rather than concentrated at a single stage, from follicular development through fertilization to post-fertilization embryo development (Wolfenson & Roth, 2019; Roth, 2017).

Embryonic and early foetal loss more broadly, whether heat-driven or not, concentrates in the first three weeks after fertilization, so a stage-resolved comparative protocol must track outcomes across this same early window rather than only a single terminal endpoint if it is to localize where a genetic effect operates (Diskin & Morris, 2008), which is precisely the reason a comparative trial that measures only one endpoint cannot characterize where a genetic thermotolerance advantage is actually expressed. At the molecular level, reactive oxygen species accumulation is a consistent correlate of reduced developmental competence in bovine embryos produced in vitro under thermal or oxidative challenge (Dalvit et al., 2005). Co-culture with granulosa cells as a monolayer has been proposed as a mitigation strategy for in vitro embryo production conducted under heat stress in a related bovid species, on the reasoning that granulosa-derived paracrine signaling can partially offset the oxidative burden of thermal challenge (Gad-Elkareem, 2021), and melatonin supplementation during culture has been shown to protect bovine embryos from the combined effects of heat stress and altered oxygen tension, improving embryo production outcomes under thermally challenging laboratory conditions (Hitit et al., 2023). Both mitigation strategies are informative for a comparative genetics trial for the same reason: if a culture-stage antioxidant or paracrine intervention can substantially narrow the gap between heat-challenged and unchallenged embryos within a single genetic group, then a between-genetic-group comparison that does not standardize these culture conditions risks attributing a laboratory-protocol effect to genetics. Oocyte developmental competence itself is acquired progressively during folliculogenesis rather than fixed at any single timepoint (Gandolfi, 1996), and a synthesis of the factors governing this competence, including donor age, breed, and metabolic state, situates thermal sensitivity as one of several interacting determinants rather than an isolated variable (Fair, 2012). Genetic variance in the fertility cost of heat stress has been demonstrated directly: quantitative genetic analysis of dairy cattle fertility traits under thermal challenge shows that heat-stress sensitivity itself carries additive genetic variance, meaning some sire and dam lines transmit greater cellular and physiological heat resilience to their offspring than others (Ojo et al., 2025). This finding is the genetic-parameter foundation for the comparative genetics claim examined in Section 2.1: if heat-stress sensitivity is heritable, a genetic group selected historically for adaptation to hot environments should be expected to show measurably different cellular thermotolerance than a genetic group selected primarily for production traits under temperate conditions, and this difference should be detectable at the level of the isolated oocyte and embryo, not only at the level of whole-animal fertility. Heat-shock protein induction is the cellular signature most directly implicated in the thermotolerance difference between genetic groups. Elevated temperature triggers rapid transcription of heat-shock protein 70 and related chaperones in the oocyte and cumulus cells, which act to stabilize partially denatured proteins and to support refolding rather than aggregation during and immediately after thermal challenge; the capacity to mount this response quickly and to sustain it without exhausting cellular energy reserves is a plausible cellular basis for a genetic-group difference in thermotolerance, since a slower or weaker heat-shock response would leave the oocyte's meiotic and cytoskeletal machinery exposed to protein misfolding for a longer window during the same thermal challenge. This mechanism is consistent with, but mechanistically distinct from, the reactive-oxygen-species pathway discussed above: heat-shock protein induction addresses protein-folding stress directly, while antioxidant and paracrine mitigation strategies address the oxidative byproduct of thermal and metabolic stress, and the two pathways may respond differently to the same heat challenge depending on genetic background. A stage-resolved comparative protocol is well positioned to test this distinction directly, since heat-shock

protein expression and reactive oxygen species accumulation can both be assayed on the same oocyte or embryo cohort at each challenge point specified in Table 1, rather than inferred indirectly from developmental outcome alone. Mitochondrial function links the heat-shock and oxidative-stress pathways described above to the specific comparative genetics evidence reviewed next. The oocyte carries a fixed mitochondrial complement established during oogenesis, with no mitochondrial replication occurring until after fertilization, so that oocyte-stage mitochondrial content and functional competence set a ceiling on the energy available for the meiotic and early cleavage divisions most vulnerable to heat-induced disruption. Reduced mitochondrial membrane potential and increased reactive oxygen species production are commonly observed together under thermal challenge, consistent with mitochondria acting as both a source and a target of heat-induced oxidative stress in the oocyte and early embryo. Because donor genetic background and donor metabolic and nutritional status both influence oocyte mitochondrial content, a comparative genetics trial that does not separately account for donor condition, as specified in Section 5, risks attributing a mitochondrial-competence difference driven by donor management to genetic ancestry, reinforcing the donor-covariate standardization requirement introduced in Section 4.

2.1 Comparative genetics: *Bos indicus* and *Bos taurus* embryo thermotolerance

The direct comparative evidence for a genetic thermotolerance advantage comes primarily from embryo-transfer and in vitro production trials that hold breed of bull, breed of cow, or both as an explicit factor. *Bos indicus*-influenced genetics show greater resistance of the early embryo to heat challenge than *Bos taurus* genetics in factorial comparisons crossing the breed of the bull with the breed of the cow, indicating that both maternal and paternal genetic background contribute independently to embryo heat resistance rather than either alone determining the outcome (Eberhardt et al., 2009). Pregnancy rate following embryo transfer differs by the breed composition of both the transferred embryo and the recipient in *Bos indicus* and *Bos indicus* by *Bos taurus* combinations, indicating that the comparative advantage documented at the cellular level in vitro is at least partially reflected in whole-animal outcome after transfer (Montiel et al., 2006), and pregnancy rates to fixed-time embryo transfer of vitrified in vitro-produced embryos differ across *Bos indicus*, *Bos taurus*, and *Bos indicus* by *Bos taurus* embryo genetic groups, extending the comparison to the cryopreserved state specifically (Marinho et al., 2015).

Large-scale comparative in vitro production work using sexed semen across *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows reports pregnancy rates that differ by genetic group at a scale large enough to be operationally, not only statistically, meaningful for a commercial in vitro production program (Pontes et al., 2010), while frozen *Bos indicus* by *Bos taurus* embryos produced from oocytes recovered from slaughtered *Bos taurus* cows demonstrate that oocyte genetic background and post-collection handling can be varied independently of the donor's own reproductive lifecycle, a design feature relevant to isolating a genetic from a management effect in a comparative trial (Hutton et al., 1997). Synchronization protocol choice has itself been shown to change fixed-time embryo transfer pregnancy rates specifically in *Bos indicus* by *Bos taurus* recipient combinations, which means protocol, not genetics alone, partially determines whether a given genetic combination achieves its expected outcome, and any comparative trial must therefore hold synchronization protocol constant across genetic groups rather than allowing it to vary with donor management convenience (Nasser et al., 2004). At the more exploratory end of this literature, diploid *Bos taurus* and tetraploid *Bos indicus* embryo aggregation has been used to produce bovine embryonic chimeras, probing the developmental compatibility of the two

ancestries at the cellular level in a way that conventional crossbreeding cannot, and indicating that the two genetic backgrounds are not simply additive even at the level of chimeric cell mixing (Montanari Razza et al., 2011). Repeat flushing for embryo recovery in crossbred donor cows further demonstrates that genetic background interacts with the number of viable embryos obtainable per donor cycle, a supply-side constraint on any comparative trial that requires matched embryo numbers across genetic groups within a limited collection window (Subramaniam et al., 1991). The mechanistic link between comparative genetics and oocyte-level cellular performance is strengthened by donor metabolic evidence. Lipid content and mitochondrial activity in oocytes collected from crossbred *Bos taurus* by *Bos indicus* cows are linked to early embryo developmental competence, indicating that the genetic-group comparison operates partly through oocyte cytoplasmic composition and mitochondrial function rather than through a single thermotolerance gene of large effect (Mendez et al., 2018), which is consistent with the heat-shock protein and oxidative-stress mechanisms reviewed above and argues against treating breed composition as a simple categorical proxy for a single underlying thermotolerance mechanism. Forty years of applied bovine in vitro fertilization work situates this comparative genetics question within the broader genomic selection context, noting that which animals are nominated as oocyte donors, and therefore which genetic backgrounds enter the comparative literature at all, has itself shifted as genomic selection tools have matured (Sirard, 2018), and genomic selection in beef cattle has created additional demand for embryo technologies capable of moving selected, including heat-adapted, genetics into commercial herds faster than natural generation intervals allow (Miller, 2022). A parallel line of applied genetics, distinct from but complementary to the *Bos indicus* ancestry-proportion measure used throughout this protocol, concerns single genes of large effect on whole-animal thermoregulation. The slick-hair phenotype, driven by a dominant mutation in the prolactin receptor gene and segregating in several *Bos taurus*-derived Criollo and Senepol-influenced populations, produces a short, sleek hair coat associated with improved evaporative cooling and lower core body temperature under heat load, and breeding programs have begun introgressing this variant into otherwise conventional dairy genetics specifically to improve thermotolerance without the production-trait tradeoffs sometimes associated with higher *Bos indicus* ancestry proportion. This paper's stage-resolved protocol is agnostic to which genetic mechanism, *Bos indicus* ancestry, a slick-hair variant, or another thermotolerance allele, underlies a given donor's classification, and the ancestry-proportion measure specified in Table 3 should be understood as one operationalization of genetic background among several that a future study could substitute or supplement, including a slick-hair genotype indicator where the relevant variant has been confirmed by genotyping, without changing the outcome cascade or challenge-point structure of the protocol itself. Taken together, this literature supports the central proposition tested by this paper's protocol: a thermotolerance advantage for environmentally adapted genetics is documented at multiple points across the in vitro production sequence, from oocyte cytoplasmic composition through embryo transfer pregnancy rate, but no single study in this literature tracks the same embryo cohort through maturation, fertilization, culture, and cryopreservation under a controlled, stage-resolved heat challenge. The comparative advantage documented at embryo transfer could in principle arise entirely from a maturation-stage effect, entirely from a culture-stage effect, or from effects at multiple stages that partially offset or compound each other; the existing literature, taken as a whole, cannot distinguish these possibilities, which is the gap this paper's protocol is designed to close.

3 Stage-resolved comparative frameworks

The framework separates the in vitro production sequence into three challenge points at which heat can be applied independently: oocyte maturation, fertilization, and post-fertilization culture. At each challenge point, environmentally adapted and conventional genetics are compared under both a thermoneutral and a heat-challenged condition, producing a two-by-three factorial design (genetic group by challenge point) crossed with a thermal condition factor, rather than the single genetic-group-by-thermal-condition comparison common in the existing literature. This design is the direct methodological response to the stage-conflation problem identified in Section 2.1: it allows a maturation-stage effect to be estimated independently of a culture-stage effect on the same underlying genetic comparison. Table 1 specifies the three challenge points, the cellular process primarily at risk at each, and the outcome measure most proximally linked to injury at that stage. Outcomes are tracked as a cascade on the same embryo cohort: cleavage is assessed on embryos that proceeded through the maturation and fertilization challenge points as specified, blastocyst formation is assessed only on embryos that cleaved, embryo grade is assigned only to embryos that reached blastocyst, and post-vitrification survival is assessed only on graded blastocysts selected for cryopreservation. This cascade structure means that a genetic-group difference at a later stage is always interpreted conditional on having survived the earlier stages, and the conditional, not only the unconditional, comparison is reported.

Table 1. Stage-resolved challenge points in the comparative in vitro production protocol.

Challenge point	Cellular process at risk	Primary outcome	Conditional on
Oocyte maturation (IVM)	Meiotic spindle organization, cumulus expansion, cytoplasmic maturation	Maturation rate (proportion reaching metaphase II)	Oocyte recovery and initial morphology grade
Fertilization (IVF)	Sperm-oocyte binding, pronuclear formation, first cleavage division	Cleavage rate at 48 hours post-insemination	Successful maturation at the prior stage
Post-fertilization culture (IVC)	Embryonic genome activation, compaction, blastulation	Blastocyst rate at day 7 to 8; embryo grade	Cleavage at the prior stage
Cryopreservation (post-IVC)	Membrane integrity and metabolic recovery after vitrification and warming	Post-warming re-expansion and survival at 24 hours	Blastocyst formation and grade at the prior stage

The genetic-group factor is operationalized as proportion Bos indicus ancestry, estimated from pedigree or, where available, genomic data, rather than as a simple binary indicus-versus-taurus label, since ancestry proportion varies continuously across the crossbred donors and bulls commonly used in commercial in vitro production programs. This continuous specification allows the comparative analysis to estimate a dose-response relationship between ancestry proportion and each stage-specific outcome, rather than assuming the relationship is a step function at some unstated ancestry threshold. Heat challenge at each stage is applied as a defined temperature elevation above standard culture temperature for a defined duration, informed by, but not assumed identical to, the thermal exposures reported in the studies reviewed in Section 2

(Ryan et al., 1992; Kasimanickam et al., 2021), since laboratory heat-challenge protocols in the existing literature vary enough in magnitude and duration that a new protocol should state its own parameters explicitly rather than citing a single prior study as though its parameters were a fixed standard. Where sufficient in vitro-produced blastocysts are available from a single genetic background, embryo splitting to produce genetically identical demi-embryos offers a within-embryo control that removes genetic variance entirely from the thermal-condition comparison at the cryopreservation challenge point, since one demi-embryo can be vitrified immediately while its genetically identical twin proceeds through an additional heat challenge before vitrification. Documented viability of split bovine embryos following fresh and frozen-thawed handling supports the feasibility of this design as a supplementary, within-genetic-group comparison, run alongside, rather than in place of, the primary between-genetic-group comparison specified in Table 1, since the two designs answer different questions: the split-embryo design isolates the pure thermal-condition effect within a fixed genetic background, while the between-group design is required to estimate the genetic-group effect that is this paper's central concern. Each stage-specific outcome in Table 1 is modeled as a binomial generalized linear mixed model, with the proportion succeeding at that stage as the response, genetic-group ancestry proportion, thermal condition, and their interaction as fixed effects, and random intercepts for donor animal and for laboratory session to absorb the batch effects discussed in Section 4. Because the cascade structure means later-stage models are fit only to embryos that survived the prior stage, the cascade as a whole is analyzed both stage-by-stage, conditional on survival to that stage, and as a single multiplicative chain from oocyte entering maturation to blastocyst surviving cryopreservation, so that a reader can recover either the conditional, stage-specific effect or the unconditional, whole-cascade probability implied by the fitted model. The genetic-group by thermal-condition interaction at each stage is the coefficient of primary interest and is reported with a profile confidence interval and, where the cascade model is used, propagated through to an implied difference in whole-cascade success probability between genetic groups under heat challenge.

4 Comparative study design and laboratory quality control

The proposed empirical design is a preregistered, multi-replicate laboratory trial using oocytes and semen from donors and sires of defined, genotyped ancestry proportion, processed in parallel through the same laboratory on the same day to control for the batch effects long recognized as a major source of variance in bovine in vitro production work. A systematic analysis of culture systems reported across five years of international embryo transfer meetings found protocol and laboratory variation to be a dominant source of reported outcome variability, larger in many comparisons than the biological effect under study (Thompson & Duganzich, 1996), which is the direct justification for running environmentally adapted and conventional genetics through identical laboratory sessions rather than comparing across published studies conducted in different laboratories at different times.

4.1 Replication, randomization, and blinding

Within each laboratory session, oocytes from environmentally adapted and conventional donors are collected, matured, fertilized, and cultured in parallel, with assignment to the thermal-challenge or thermoneutral condition randomized within genetic group rather than run as two sequential batches, since sequential running would confound thermal condition with any drift in incubator performance, medium batch, or technician fatigue over the course of a session. Embryo

and blastocyst morphological grading, the step in the outcome cascade most subject to human judgment, is performed by graders blinded to genetic group and thermal condition wherever the physical appearance of the embryo does not itself reveal the assignment; where blinding is not fully achievable, for example because heat-challenged embryos are visibly different from thermoneutral embryos at grading, this limitation is stated explicitly rather than presented as though full blinding had been achieved. A second, independent grader scores a random subset of embryos at each session to allow inter-grader agreement to be reported as a formal reliability statistic rather than assumed. Technician identity is recorded and entered as a candidate random effect alongside laboratory session, since technician and session are often, though not always, confounded on smaller operations, a limitation that should be stated rather than concealed by folding technician variance entirely into the session random intercept.

4.2 Sample size and statistical power

The factorial design crossing genetic group, thermal condition, and challenge point requires a power calculation anchored to the interaction terms specified as hypotheses H1 through H3, not to the simpler main effect of thermal condition on any single outcome. Detecting a genetic-group by thermal-condition interaction of a given standardized magnitude typically requires several times the number of replicate oocytes or embryos needed to detect a main effect of the same magnitude, and the donor- and session-level clustering built into the mixed model further inflates the effective variance through a design effect proportional to the intraclass correlation of outcomes within donor and within session. The power calculation should therefore be performed at the level of the donor-by-session cell, using intraclass correlation estimates from prior in vitro production datasets where available, and should report the minimum detectable interaction effect at the planned number of sessions and donors per genetic group rather than reporting only a total oocyte or embryo count, since the same total count distributed across many small sessions and distributed across few large sessions yields materially different effective power once clustering is accounted for. Because the outcome cascade narrows at each successive stage, from oocytes entering maturation to blastocysts entering cryopreservation, the power available to detect a genetic-group effect on post-warming survival is necessarily lower than the power available at maturation, for a fixed starting number of oocytes. The protocol therefore requires that the planned oocyte number be set with reference to the power requirement at the least frequent, most downstream outcome of interest, typically post-warming survival, rather than with reference to the power requirement at maturation rate, which would be met first but would leave the cascade underpowered at the stage most relevant to commercial application. A related consideration is the timing of donor and sire enrollment relative to the collection schedule. Enrolling all donors in both genetic groups before any collection begins, rather than enrolling opportunistically as animals become available across the collection season, avoids a secular confound in which one genetic group is disproportionately represented in early, cooler-season sessions and the other in late, warmer-season sessions, which would bias the thermal-condition comparison independent of the controlled heat challenge applied in vitro. Where full advance enrollment is not feasible, session date and ambient conditions at the donor's home location should be recorded and entered as covariates, so that any residual seasonal imbalance across genetic groups can be detected and adjusted for rather than left unexamined. Interim monitoring across the planned collection sessions should use a prespecified stopping rule rather than informal inspection of accumulating results, since repeated informal looks at an accumulating genetic-group comparison inflate the false-positive rate for the primary hypothesis in the same way that repeated peeking inflates error

rates in a clinical trial. Where feasible, an analyst independent of the laboratory processing the embryos should conduct any interim look, with the outcome of that look limited to a decision about whether to continue, extend, or stop collection, and with no interim effect estimate disclosed to the embryologists or graders performing ongoing blinded assessment.

Maturation medium, fertilization medium, and culture medium composition, including carbon dioxide concentration, are held constant across genetic groups within a replicate, since medium composition and carbon dioxide concentration materially change developmental rates in bovine in vitro production systems (Merton et al., 1995), and serum and cysteamine supplementation choices have been shown to change cleavage rate specifically, independent of genetic background (Sandal, 2020). Sperm treatment and insemination conditions, including recombinant gonadotropin protocols used to stimulate donors where applicable, are likewise standardized across genetic groups (Brackett & Keskinetepe, 1996; Martins et al., 1998), and plasminogen activator production by the early embryo, linked mechanistically to developmental progression in vitro, is recorded as a process-level covariate rather than assumed uniform across genetic groups (Kaaekuahiwi & Menino, 1990). Genetic parameters for oocyte number and embryo production within ovum-pickup-based programs indicate that donor-level genetic merit for oocyte yield is itself heritable and selectable, independent of the thermotolerance comparison under study, and is therefore recorded as a separate donor-level covariate (Merton et al., 2009). A comprehensive review of oocyte recovery, in vitro production, and embryo transfer status in domestic cattle provides the procedural baseline against which laboratory competence for this comparative protocol should be benchmarked before a genetic comparison is attempted (Hasler, 1998). Embryo and oocyte selection methods applied uniformly across genetic groups include morphological selection of immature oocytes for developmental potential before maturation, a method shown three decades ago to change downstream blastocyst yield independent of genetic background (Madison, Avery, & Greve, 1992), and more recent staining-based selection methods, such as brilliant cresyl blue assessment of oocyte metabolic competence, which refine the same selection principle (Goktepe Turhan & Cetin, 2025). Molecular markers of oocyte competence extend selection beyond morphology: recombinant bone morphogenetic protein 15 targets a signaling pathway intrinsic to oocyte-cumulus communication and has been tested specifically for its capacity to improve bovine in vitro embryo production systems (Burns et al., 2010). Where embryo splitting is used to produce genetically identical demi-embryos for parallel thermal and thermoneutral assignment within the same genetic background, viability following fresh and frozen-thawed handling of split embryos has been documented and informs the feasibility of this within-embryo control strategy (Imron et al., 2012). Inadvertent sex selection has been documented as an unintended consequence of standard in vitro bovine embryo production protocols, indicating that embryo sex ratio can vary systematically by protocol; sex ratio is therefore recorded and reported by genetic group and thermal condition rather than assumed balanced by default (Behboodi et al., 1997). Polar body genetic diagnosis has been proposed as a selection tool for identifying euploid oocytes before insemination, and polar body array comparative genomic hybridization, debated in the human assisted-reproduction literature for its accuracy as an embryo-selection method, offers a conceptual parallel for genetic screening that the bovine field has not yet resolved with an equivalent validation standard (Fabres et al., 2012; Shulman, 2012; Scriven et al., 2012); where genetic screening is applied to select embryos for this trial's genetic-group comparison, the unresolved validation status of the screening method itself should be reported as a limitation rather than treated as ground truth.

Cryopreservation procedure is standardized across genetic groups using a single vitrification protocol, since donor blood cholesterol has been linked to embryo color and freezability, tying donor metabolic status to post-thaw viability independent of genetic ancestry (Hill & Kuehner, 1998); donor cholesterol status is therefore recorded as a covariate so that a genetic-group difference in post-warming survival is not confounded with an unmeasured difference in donor metabolic status across the two genetic groups being compared. A broader review of oocyte recovery, in vitro production, and embryo transfer status in domestic animals situates cryopreservation as the final and commercially decisive stage of the outcome cascade specified in Table 1, reinforcing why post-warming survival, not only blastocyst rate, is retained as a primary endpoint in this protocol (Hoshi, 2003). Field and industry standards for embryo transfer procedure, established by veterinary professional bodies, provide the procedural baseline for the post-thaw handling and transfer steps that lie downstream of this paper's laboratory-level comparison (American Association of Bovine Practitioners, 1985). Environmental contaminant exposure in the water used for wash and culture media preparation is a further covariate worth recording explicitly, given documented variation in per- and polyfluoroalkyl substance contamination across water systems and its association with adverse developmental outcomes in other species (Oyelade & Ohanebo, 2024; Oyelade & Ohanebo, 2025); a comparative trial that draws laboratory water from a contaminated source without recording this exposure risks attributing a water-quality effect to genetic background if contamination happens to correlate with laboratory location or donor source. Prediction and selection serve different purposes and should not be conflated in how this trial's results are used downstream. A predictive model estimates outcomes for new oocytes or embryos; a selection index combines genetic merit across traits using economic or biological weights; both require external validation and safeguards against overinterpretation of a single trial (Meuwissen et al., 2001), and the logic of multi-trait selection, formalized in the original selection-index literature, underlies how donor animals are nominated for comparative in vitro production trials in the first place, even though this paper's purpose is stage-resolved comparison rather than index construction (Hazel, 1943).

4.3 Data governance and reproducibility

Every oocyte, embryo, and outcome record enters an immutable raw data layer at the point of observation, tagged with donor identity, session identifier, grader identity, and timestamp, before any curation or exclusion is applied. A versioned curated layer applies range and consistency checks, for example flagging a recorded blastocyst rate that exceeds the cleavage rate for the same cohort as a data-entry error requiring resolution before analysis rather than silently correcting it. The locked analysis dataset used for the primary genetic-group comparison is frozen before that comparison is run, with any post hoc correction documented as a new, dated dataset version rather than an undocumented edit to the original. Embryo photographs and, where available, time-lapse image sequences are retained as primary evidence supporting each morphological grade, so that a disputed or borderline grading decision can be independently re-reviewed rather than relying solely on the recorded ordinal score. This retention standard is what allows the inter-grader reliability statistic specified in Section 5.2 to be audited after the fact rather than taken on faith.

5 Outcome cascade and donor-management covariates

Table 2 specifies the outcome cascade in sequence with the numerator, denominator, and assessment window for each measure, so that a stage-specific genetic-group effect can be

reported without ambiguity about what proportion of what starting population each rate represents.

Table 2. Outcome cascade: definitions, denominators, and assessment windows.

Outcome	Numerator	Denominator	Assessment window
Maturation rate	Oocytes reaching metaphase II	Oocytes entering IVM	22 to 24 hours post-collection
Cleavage rate	Presumptive zygotes with 2 or more cells	Oocytes entering IVF	44 to 48 hours post-insemination
Blastocyst rate	Embryos reaching blastocyst stage	Cleaved embryos entering IVC	Day 7 to day 8 post-insemination
Embryo grade	Embryos scored grade 1 or 2 on standard morphological scale	Blastocysts formed	Day 7 to day 8, at grading
Post-warming survival	Blastocysts re-expanding and surviving to 24 hours post-warming	Blastocysts vitrified	24 hours post-warming

Donor-side body condition and nutritional status are recorded as covariates because they determine oocyte yield and quality independent of, and prior to, any genetic-group thermotolerance comparison. Breed, age, body condition score, and nutritional status jointly predict follicular population, oocyte yield, and oocyte quality in zebu cattle, establishing body condition as a donor-side variable relevant to this trial regardless of genetic group (Dorice et al., 2019), and body condition score is independently associated with production performance in Sahiwal cattle, a *Bos indicus* breed frequently used as a donor source in heat-adapted comparative work (Sharma, 2020; Singh et al., 2020). Invited review evidence on body condition score and dairy cow productivity, and subsequent work relating body condition to reproductive performance and body weight in seasonal-calving systems, together establish body condition as a general physiological readout that should be standardized or statistically controlled across donor groups in any comparative reproductive trial (Roche et al., 2009; Roche et al., 2007). Parity and body condition jointly predict pregnancy rate in beef cattle (Rae et al., 1993), and body condition score covaries with milk yield and calf birth weight (Kayar & Erzurum, 2025), crossbreeding history and postpartum disease incidence (Rinell & Heringstad, 2018), and lameness and lactation-stage confounds (Ozturan & Akin, 2025), together indicating that body condition score should be interpreted alongside a basic donor health and production history rather than recorded as an isolated number. Genetic analysis of body condition score itself confirms the trait is heritable and genetically correlated with productivity and locomotion measures in dairy cattle, which means donor body condition trajectory is partly a genetic property of the donor and should be reported as such rather than attributed entirely to management (Weller & Ezra, 2025; Liu, Yi, & Yang, 2013; Nephawe et al., 2004). Automated, image-based body condition scoring allows this covariate to be captured repeatedly across the donor pool at a cost previously prohibitive for a multi-replicate comparative trial (Liu, He, & Norton, 2020), and standardized visual scoring protocols provide the measurement basis needed to apply this covariate consistently where automated imaging is not available (Hersom & Thrift, 2018). Documented confounds, such as indigestible foreign objects in the gastrointestinal tract altering apparent body condition

independent of nutritional status, are a further reason body condition should be interpreted alongside a basic health screen (Nongcula et al., 2017).

Forage-based nutritional management of the donor herd is recorded as a second covariate layer, since forage dry matter intake, energy density, and protein degradability determine the substrate available for folliculogenesis independent of genetic ancestry. Classic ensiling and forage-quality trials establish that dry matter content, fiber fraction, and processing method jointly determine intake and production response in cattle (Keady, Murphy, & Harrington, 1996; Forbes & Jackson, 1971; Keady & Steen, 1994; Keady & Steen, 1995), and chemical and physical forage characteristics were identified three decades ago as primary determinants of intake independent of simple dry matter percentage (Sekine et al., 1995a; Sekine et al., 1995b). Protein and dry matter degradability in the rumen differ systematically across forage-to-concentrate ratios and across cattle, buffalo, and sheep (Terramoccia et al., 2000), which is directly relevant to a comparative donor herd because *Bos indicus*-influenced and *Bos taurus* donors on the same forage base can differ in intake and digestion kinetics independent of any reproductive thermotolerance difference. Forage yield and quality are themselves environmentally contingent, varying with manure and fertilization regime (Yoottasano et al., 2015; Oyharcabal et al., 2024) and with grazing selection behavior under heat and drought (Katjiua & Ward, 2006), so the donor-herd forage covariate should be recorded as a season-linked exposure rather than a fixed ration specification, consistent with the environmental-exposure framing used elsewhere in this research program. Table 3 consolidates the donor-management and laboratory covariates specified above into a single measurement reference, so that a laboratory implementing this protocol can verify, before a genetic-group comparison begins, that each covariate has a defined source, unit, and recording point.

Table 3. Donor-management and laboratory covariates recorded alongside the genetic-group comparison.

Covariate	Definition and unit	Recording point	Rationale
Ancestry proportion	Proportion <i>Bos indicus</i> ancestry, 0 to 1, pedigree or genomic estimate	Once, at donor and sire enrollment	Primary comparative exposure; continuous, not binary
Donor body condition score	1 to 5 scale (quarter-point)	At each collection session	Oocyte yield and quality confound independent of genetics
Donor forage dry matter intake	kg DM per head per day, herd or group estimate	Continuous, donor herd management record	Energy substrate for folliculogenesis
Laboratory session	Categorical session identifier	Every processing batch	Captures medium, incubator, and day-to-day protocol drift
Technician / grader identity	Categorical identifier, separate from session where feasible	Every procedure and every grading event	Captures operator-dependent variance
Water-source contaminant status	Categorical: tested and below reference level, tested and above, or	Annual or as tested	Confound for wash and culture media preparation

	untested		
Donor cholesterol / metabolic status	Serum cholesterol, mg per dL, at collection	At each collection session where feasible	Linked to embryo colour and freezability independent of ancestry

5.2 Reliability of morphological and imaging endpoints

Standard morphological grading of blastocysts on an ordinal scale carries known inter-observer variability, and the protocol requires that inter-grader agreement be reported using a chance-corrected agreement statistic on the blinded double-graded subset described in Section 4.1, rather than assumed adequate because graders were trained using a common reference atlas. Where agreement falls below a prespecified threshold, grading criteria should be recalibrated across graders before the genetic-group comparison is finalized, and the achieved agreement statistic should be reported alongside the primary result rather than omitted as a methods detail. Emerging time-lapse imaging systems, which record continuous morphokinetic parameters such as time to first cleavage and time to blastulation rather than a single endpoint grade, offer a path to reducing this observer dependency, and where available should be used to supplement, though not necessarily replace, morphological grading, since morphokinetic parameters may themselves differ by genetic group in ways a single terminal grade would not capture.

Genomic estimation of ancestry proportion, where donor and sire genotyping is available, carries its own reliability statistic tied to reference-population size and marker density, and this reliability should be retained and reported alongside the point estimate of ancestry proportion rather than discarded once a single ancestry value is assigned. A donor whose ancestry proportion is estimated from a small or poorly characterized reference population should not be treated as equally precisely classified as a donor estimated from a large, well-validated reference population, and the statistical model specified in Section 3 should, where feasible, downweight or flag lower-reliability ancestry estimates rather than treating every donor's ancestry-proportion value as measured without error. Maturation rate assessment depends on correct identification of metaphase II arrest by either polar body extrusion under light microscopy or, more precisely, confirmed spindle configuration under polarized light or fluorescent staining; the two methods carry different sensitivity to borderline cases, and the protocol requires that the method used be reported and held constant across genetic groups within a session, since a method more sensitive to borderline maturation could otherwise be applied differentially in a way that manufactures an apparent genetic-group difference. Laboratory batch and technician effects, already discussed as a source of variance in the broader in vitro production literature, apply with equal force to maturation and cleavage assessment and are captured through the session and technician random effects specified in the statistical model above.

6 Testable hypotheses

Six hypotheses follow from the cellular mechanisms in Section 2, the comparative genetics evidence in Section 2.1, and the stage-resolved design in Section 3.

H1 (stage specificity). The thermotolerance advantage of higher *Bos indicus* ancestry proportion is larger, in standardized effect-size terms, at the maturation challenge point than at the culture challenge point. This hypothesis directly tests the stage-conflation problem identified in Section 2.1 and is the paper's primary confirmatory test.

H2 (cascade attenuation). The genetic-group difference in blastocyst rate, conditional on cleavage, is smaller than the unconditional genetic-group difference in cleavage rate, indicating

that some but not all of the maturation- and fertilization-stage advantage propagates forward to blastocyst formation.

H3 (cryotolerance dissociation). Genetic-group ranking on post-warming survival does not necessarily match genetic-group ranking on blastocyst rate, reflecting the donor-cholesterol and membrane-composition evidence reviewed in Section 4 that cryotolerance depends on metabolic factors partially independent of developmental competence up to the blastocyst stage.

H4 (donor condition interaction). The genetic-group effect on oocyte yield and maturation rate is larger among donors in the lowest body-condition-score tertile than among donors in the highest tertile, indicating that any thermotolerance advantage is partially masked under favorable donor management and more visible under donor nutritional restriction.

H5 (mitigation interaction). Culture-stage antioxidant or paracrine mitigation, such as granulosa co-culture or melatonin supplementation, narrows the genetic-group gap in blastocyst rate under heat challenge, indicating that at least part of any observed genetic-group difference reflects a modifiable cellular stress response rather than a fixed, non-modifiable genetic ceiling.

H6 (protocol sensitivity). The magnitude and even the direction of the genetic-group effect at each challenge point is sensitive to the specific heat-challenge temperature and duration used, such that results from this protocol should not be extrapolated to a different challenge magnitude without re-validation. This hypothesis is deliberately self-limiting: it constrains how the paper's own eventual results should be interpreted and generalized.

As with any comparative genetics claim, a null or reversed result at a given stage is treated as informative rather than as a failed experiment. A null result at the maturation challenge point, combined with a positive result at the culture challenge point, would itself be a finding, indicating that the thermotolerance advantage reported in prior embryo-transfer literature operates later in the production sequence than commonly assumed, and would redirect future mitigation research toward culture-stage rather than maturation-stage intervention.

6.1 Sequencing of hypothesis tests

H1 is the primary confirmatory test and carries a single preregistered significance threshold, given the direct mechanistic grounding provided by the heat-shock protein and comparative embryo-transfer evidence reviewed in Sections 2 and 2.1. H2 and H3 are secondary confirmatory tests concerning cascade propagation and cryotolerance dissociation respectively, tested at a threshold adjusted for the two comparisons and reported with the conditional sample size available at each downstream stage, which will necessarily be smaller than the starting oocyte count given the narrowing cascade described in Section 4.2. H4 and H5 are interaction hypotheses concerning donor management and culture-stage mitigation and are reported descriptively alongside their formal test, since their practical interpretation, described in Section 7, depends on the direction and magnitude of the interaction rather than on statistical significance alone. H6 is a scope-limiting rather than a confirmatory hypothesis and does not compete with the others for significance budget; it exists to bound how the study's own results, once obtained, should and should not be generalized to a different heat-challenge magnitude. This sequencing mirrors the logic used elsewhere in this research program: a single, well-grounded primary hypothesis is protected from dilution by simultaneous testing of several lower-priority comparisons, while the lower-priority hypotheses are still reported in full rather than discarded, since a null result on a secondary hypothesis remains informative for the stage-specificity question even when it is not the paper's primary confirmatory claim.

7 Reporting standards and translational pathway

A stage-resolved comparative trial is only as useful as its reporting allows a reader to reconstruct. The protocol requires that every published or internal report state, for each genetic group and each challenge point, the exact temperature elevation and duration applied, the medium composition and carbon dioxide concentration used, the donor body condition score and forage management context, and the number of replicates run on the same day in the same laboratory session. Reporting a pooled blastocyst rate across replicates run in different laboratory sessions, without a session random effect in the statistical model, is treated as a reporting deficiency regardless of the significance of the reported genetic-group effect, given the batch-effect evidence reviewed in Section 4. Translation from laboratory finding to breeding-program practice should proceed through the same staged logic used elsewhere in this research program: a definition and readiness stage establishing donor and protocol standards; a pilot stage confirming that the stage-resolved design is executable within a given laboratory's throughput and quality-control capacity; a protected evaluation stage replicating the comparison across multiple donor cohorts and, where feasible, multiple laboratories, to test whether a stage-specific effect observed in one laboratory transfers to another; and only then a decision-support stage in which a validated stage-specific thermotolerance profile informs which genetic group, or which culture-stage mitigation, a commercial in vitro production program adopts for use under heat-stressed field conditions. A single-laboratory, single-cohort result, however large the observed effect, does not on its own justify a change in commercial donor selection or culture protocol.

The decision-value logic set out above depends on an explicit accounting of relative cost, not only relative effect size. Donor genetic selection is a slow, herd-level investment realized only after a generation interval, while culture-stage antioxidant supplementation is a fast, per-session investment realized immediately but recurring indefinitely; a small but statistically confirmed genetic-group advantage may still be the more cost-effective long-run investment even if a larger short-run gain is available from culture-stage mitigation, once the recurring cost of mitigation is amortized across the program's expected operating horizon. The protected-evaluation stage should therefore report not only the statistical magnitude of each stage-specific effect but also a simple cost-per-additional-surviving-blastocyst comparison across the genetic-selection and culture-mitigation pathways, so that a program can weigh the two investments on a common basis rather than on statistical significance alone.

Where a program lacks the throughput to run the full factorial design in Table 1 within a single collection season, a staged reduction is preferable to an uncontrolled shortcut: the maturation and culture challenge points, hypothesized in H1 to carry the largest genetic-group effect, should be prioritized over the fertilization challenge point if a choice must be made, and the cryopreservation endpoint should never be dropped entirely even under a reduced design, since it is the endpoint most directly tied to the commercial decision the study is ultimately meant to inform. A reduced design that preserves the full outcome cascade at fewer challenge points is preferable to a full-factorial design that stops recording outcomes before cryopreservation.

None of these stages is meaningful without embryologist and technician capacity building, since the grading, staining, and cryopreservation procedures underlying the outcome cascade are only as reliable as the training of the personnel performing them. The readiness stage should include a documented training and certification protocol for each manually performed procedure, with a scheduled recalibration interval and a minimum caseload requirement before a technician's grading is included in the primary comparative dataset, rather than treating training as a one-time credential that does not require ongoing verification across a multiyear, multisession trial.

The decision value of a validated stage-specific thermotolerance profile lies in directing limited laboratory and breeding-program investment toward the stage where it will do the most good. If H1 is confirmed and the advantage is concentrated at maturation, investment in donor selection for thermotolerant genetics yields more than investment in culture-stage antioxidant supplementation, since the culture-stage intervention would be acting downstream of where most of the damage has already occurred. If instead the advantage proves concentrated at culture, as tested by the inverse of H1, culture-stage mitigation strategies become the more cost-effective investment regardless of donor genetic background, and a program need not restrict itself to environmentally adapted donors to achieve most of the available benefit. Reporting the outcome cascade in full, rather than a single pooled endpoint, is what allows this investment decision to be made on stage-specific rather than aggregate evidence.

8 Discussion

The cellular and comparative genetics literature reviewed in Sections 2 and 2.1 converges on a claim narrower, and more useful, than the broad statement that heat-adapted genetics outperform conventional genetics under heat stress: the advantage is documented at multiple stages and multiple endpoints, but not shown, within any single existing study, to be uniform across the full production sequence from maturation through cryopreservation. The practical implication for an in vitro production laboratory is that a genetic-group comparison conducted at only one stage, most commonly blastocyst rate at day 7 or 8, risks either overstating or understating the advantage that would be observed if the same embryos were tracked through cryopreservation and post-warming survival, which is the endpoint most directly tied to commercial value.

The donor-management covariate evidence carries a second implication. Because body condition and forage management determine oocyte yield and quality independent of genetic ancestry, a comparative trial that sources environmentally adapted donors from a lower body-condition population and conventional donors from a higher body-condition population, whether by design or by the practical availability of donors, will confound genetic-group and donor-management effects in a way that favors the better-managed group regardless of underlying genetics. Hypothesis H4 exists specifically to test whether this confound would matter in practice, but the safer design choice, specified in Section 4, is to standardize or statistically control donor body condition and forage management across genetic groups from the outset rather than to rely on a post hoc interaction test to detect the confound after the fact. The mitigation evidence reviewed in Sections 2 and 4, including granulosa co-culture, melatonin supplementation, and culture-medium optimization, raises a further question this protocol is designed to answer rather than assume: if culture-stage mitigation can narrow the gap between heat-challenged and unchallenged embryos within a genetic group, does it narrow the gap between genetic groups by a comparable amount, or does the environmentally adapted group's advantage persist even under mitigation? A positive answer to the second framing would indicate a genuinely genetic, rather than purely modifiable-stress-response, basis for the comparative advantage, with direct implications for whether breeding programs should prioritize genetic selection for thermotolerance or laboratory-protocol investment in mitigation, or, more plausibly, some combination calibrated to each stage's relative contribution to the overall outcome cascade.

The reliability standards specified in Section 5.2 anticipate a broader shift already underway in commercial embryology, in which time-lapse morphokinetic imaging and automated image-based grading are beginning to supplement, and in some laboratories replace, single-timepoint manual grading. A comparative protocol specified only in terms of a manually assigned ordinal

grade risks becoming obsolete as this technology diffuses, while a protocol specified in terms of the underlying developmental construct, such as blastocyst formation and re-expansion capacity, remains valid regardless of whether the construct is measured by manual grading or by continuous morphokinetic tracking. The outcome cascade in Table 2 is written at the level of the construct for this reason, with the measurement method itself recorded as a covariate rather than assumed fixed, so that laboratories at different stages of imaging technology adoption can still contribute comparable data to the same underlying comparison.

Access to in vitro production technology itself is not uniform across the operations that would benefit most from a validated stage-specific thermotolerance profile. Ovum pick-up and in vitro production infrastructure remain concentrated in well-resourced commercial and research settings, while many pastoral and smallholder systems that rely most heavily on Bos indicus-influenced genetics for thermotolerance have the least access to the laboratory capacity needed to apply IVP-derived findings directly. A stage-specific finding that culture-stage mitigation, rather than donor genetic selection, drives most of the observed advantage would be of limited practical value to a system without access to controlled culture facilities at all; the translational pathway in Section 7 should therefore report findings in a form usable by natural-service and artificial-insemination-based systems, such as which donor-side or sire-side genetic markers correlate with the laboratory-confirmed thermotolerance profile, not only in a form that presumes in vitro production access. A validated stage-specific thermotolerance profile also feeds a decision this paper does not itself make: whether and how heavily to weight thermotolerance against production traits when nominating donor animals for a breeding or embryo-production program. That weighting question belongs to multi-trait selection-index construction, with its own economic weighting, genetic-correlation, and correlated-response logic, addressed in a companion protocol rather than here. This paper's findings would enter that index-construction process as an input, specifically as evidence on the genetic correlation between ancestry proportion and stage-specific embryo performance under heat challenge, but the index weighting itself, and the question of how much expected genetic gain in production traits a breeding program should trade for a given gain in embryo-level thermotolerance, is deliberately left to that separate analysis. A related boundary concerns semen and sire-side contributions to embryo thermotolerance, which this protocol records as a donor-side ancestry and reliability covariate but does not treat as an independent comparative axis in its own right. Sperm treatment and insemination conditions are standardized across genetic groups in Section 4 specifically so that a sire-side effect does not masquerade as an oocyte-side genetic-group effect, but a full factorial comparison crossing sire genetic background with oocyte genetic background, analogous to the bull-breed by cow-breed factorial reviewed in Section 2.1, would require a substantially larger design than specified here and is left to a dedicated extension of this protocol rather than folded into the present three-factor design. This paper's scope is deliberately confined to the oocyte and embryo, and it does not extend to recipient-heifer pregnancy outcome, which depends on uterine and endocrine factors, including body condition, nutrition, and thermal exposure of the recipient rather than the donor, addressed in a companion protocol focused on predictive modeling of pregnancy success. The two protocols are complementary rather than overlapping: this paper isolates the genetic and laboratory-protocol contribution to embryo quality before transfer, while the companion protocol addresses what happens after a competent embryo, or a natural-service pregnancy, is placed in a given recipient's uterine environment. Neither protocol alone can fully explain whole-program pregnancy rate, and a research program relying on only one would risk attributing a recipient-side failure to embryo genetics, or an embryo-side failure to recipient

management. A further methodological implication concerns how future systematic reviews should treat this comparative literature. A meta-analysis that pools blastocyst rates across studies conducted at different challenge stages, different heat-challenge magnitudes, and different donor management standards, without coding for these design features as moderators, will produce a pooled effect estimate that answers no single well-defined question. The reporting checklist implicit in Tables 1 through 3, covering challenge stage, challenge magnitude, donor condition, and laboratory session structure, is offered as a minimum coding scheme for any future systematic review of this literature, not only as a data-collection standard for new primary studies. Finally, the split-embryo design described in Section 3 and the donor-covariate standardization described in Section 4 together address a criticism that could otherwise be raised against any laboratory-based comparative genetics claim: that an observed difference reflects uncontrolled donor management or laboratory handling rather than genetics as such. By combining a between-genetic-group design, controlled for donor condition and laboratory session, with a supplementary within-genetic-group split-embryo design that removes genetic variance entirely from the thermal-condition comparison, the protocol allows a reader to triangulate between two independent lines of evidence rather than relying on either design alone. Convergent findings across both designs would constitute considerably stronger evidence for a genuine genetic effect than either design could provide in isolation.

The framework's limitations follow from its status as a design rather than a completed trial. No cleavage rate, blastocyst rate, embryo grade distribution, or post-warming survival figure is reported here because none has been produced under this protocol; every quantitative claim in Sections 3 through 6 is a hypothesis with a specified test, not a result. The contribution lies in specifying the stage-resolved design precisely enough that a future trial, conducted under appropriate laboratory and animal-care oversight, can be evaluated against a preregistered plan rather than against outcomes selected after the embryos were examined.

9 Conclusion

Comparing in vitro fertilization and embryo development performance between environmentally adapted and conventional cattle genetics under heat stress requires resolving, rather than averaging across, the stage at which a genetic effect is expressed. This paper has specified a stage-resolved protocol that separates maturation, fertilization, and culture challenge points; tracks a linked outcome cascade from cleavage through post-warming survival on the same embryo cohort; standardizes laboratory protocol and donor management as covariates rather than uncontrolled sources of variance; and states six falsifiable hypotheses about where and how strongly a thermotolerance advantage for environmentally adapted genetics is expressed. The central claim is that the existing comparative literature, while consistent in direction, has not resolved the stage-specificity question this protocol is designed to answer, and that a pooled, single-endpoint comparison risks misattributing a stage-specific effect to genetics as a whole. The paper's variable-level specifications, in Tables 1 through 3, are intended to function as a shared reporting standard even for laboratories that cannot implement the complete factorial design: reporting challenge stage, challenge magnitude, donor ancestry proportion, donor body condition, and laboratory session identity alongside any subset of the outcome cascade would already improve comparability across the field relative to current practice, in which these design features are frequently left unstated. Future laboratory work should apply this protocol under appropriate institutional animal-care and biosafety oversight, report the full outcome cascade rather than a single endpoint, and treat a null result at any given stage as informative about where

thermotolerance mechanisms do, and do not, operate. If the advantage proves concentrated at maturation, breeding and donor-selection programs gain the stronger justification; if it proves concentrated at culture, laboratory-protocol investment in mitigation gains the stronger justification; and if it proves genuinely distributed across the full sequence, as the existing pooled-endpoint literature would predict, both investments are warranted together rather than as substitutes for one another. Resolving which of these patterns holds is squarely an empirical question this protocol, not the present paper, is positioned to answer.

Declarations

Ethics approval and consent to participate: This manuscript is a comparative laboratory protocol and framework paper; no oocytes, semen, or embryos were collected or processed for it. A future empirical study implementing this protocol must obtain institutional animal care and use committee approval for donor and sire animal handling and sample collection, and must obtain the consent of participating herd owners for use of donor animals and records, before any biological material is collected.

Consent for publication: Not applicable.

Availability of data and materials: No new laboratory dataset was generated or analyzed for this paper. The stage-resolved protocol, outcome cascade definitions, and covariate specifications required to reproduce the proposed comparative trial are reported in full within the manuscript.

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